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Novel Roles for Reelin in Retinogeniculate Targeting

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NOVEL ROLES FOR REELIN IN RETINOGENICULATE TARGETING

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

by

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TABLE OF CONTENTS

Acknowledgements.....	ii
List of Tables.....	iv
List of Figures.....	v
Abbreviations.....	vi
Abstract.....	ix
Introduction.....	1
Materials and Methods.....	30
Results.....	40
Reelin protein expression is high in the vLGN and IGL.....	40
RGC projections to the vLGN and IGL are diminished in the reeler mutant.....	42
ipRGCs are morphologically normal in reeler mutants.....	46
ipRGC axon targeting to the vLGN and IGL is disrupted in the reeler mutant....	48
Image forming RGCs and their dLGN projections are normal in the reeler mutant	48
Layer VI corticothalamics to dLGN are normal in reeler.....	49
Cytoarchitecture of the LGN is normal in reeler.....	50
Discussion.....	80
References.....	90

List of Tables

Table 1: Antibodies.....	31
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List of Figures

	Page
<i>Introduction</i>	
Figure 1: Synapse Formation.....	16
Figure 2: Eye Anatomy.....	18
Figure 3: Retinal Cell Types.....	20
Figure 4: Retinogeniculate Projections.....	22
Figure 5: Preliminary Experiments.....	24
Figure 6: Timeline of Retinogeniculate Development.....	26
Figure 7: Reelin Pathway.....	28
 <i>Methods</i>	
Figure 8: Measurement of Retinogeniculate Projections.....	39
 <i>Results</i>	
Figure 9: Reelin protein expression is highest in vLGN and IGL at young ages.....	52
Figure 10: P14 RGC projections to LGN	54
Figure 11: Misrouting of axons away from reeler mutant LGN.....	56
Figure 12: Rostral to Caudal Series of Wild Type and Mutant LGN.....	58
Figure 13: Mutant RGC projections to LGN compared to control.....	60
Figure 14: P1 retinogeniculate projections are altered in the reeler mutant.....	62
Figure 15 Melanopsin expressing intrinsically photosensitive retinal ganglion cells (ipRGCs), appear normal in the absence of reelin	64

Figure 16: Melanopsin expressing ipRGCS display normal morphology in the absence of reelin	66
Figure 17: RGC projections to the Olivary Pretectal Nucleus (OPN) and Suprachiasmatic Nucleus(SCN) appear normal in the reeler mutant.....	68
Figure 18: M1 retinogeniculate projections are altered in the absence of reelin	70
Figure 19: Calretinin expressing, image-forming RGCs, appear normal in the absence of reelin.....	72
Figure 20: Calretinin expressing RGCs normally target the dLGN in reeler mutants.....	74
Figure 21: Corticothalamics originating from Layer VI correctly target dLGN in reeler mutants	76
Figure 22: Immunohistochemistry of P17 LGN Cytoarchitecture.....	78

Abbreviations

ApoER2.....	Apolipoprotein Receptor 2
Calr.....	Calretinin
CNS.....	Central Nervous System
CSPG.....	Chondrotin Sulfate Proteoglycan
CTB.....	Cholera Toxin B
Dab1.....	Disabled-1 Gene
DAPI.....	4',6-diamidino-2-phenylindole
dLGN.....	Dorsal Lateral Geniculate Nucleus
DMSO.....	Dimethyl sulfoxide
E(#).	Embryonic Day
ECM.....	Extracellular Matrix
GCL.....	Ganglion Cell Layer
IGL.....	Intergeniculate Leaflet
IHC.....	Immunohistochemistry
INL.....	Inner Nuclear Layer
ipRGC.....	Intrinsically Photosensitive Retinal Ganglion Cell
LGN.....	Lateral Geniculate Nucleus
M1, M2.....	Classes of melanopsin expressing cells
mRNA.....	Messenger Ribonucleic Acid
NaOH.....	Sodium Hydroxide
OC.....	Optic Chiasm
ON.....	Optic Nerve

ONL.....	Outer Nuclear Layer
OPL.....	Outer Plexiform Layer
OPN.....	Olivary Pretectal Nucleus
OT.....	Optic Tract
P(#).	Postnatal Day
PBS.....	Phosphate Buffered Saline
PFA.....	Paraformaldehyde
QPCR.....	Quantitative Real Time Polymerase Chain Reaction
RGC.....	Retinal Ganglion Cell
SC.....	Superior Colliculus
SCN.....	Suprachiasmatic Nucleus
Sema.....	Semaphorin
Shh.....	Secreted Sonic Hedgehog
TAE.....	tris-acetate buffer with EDTA
Tris-HCL.....	tris(hydroxymethyl)aminomethan-hydrochloric acid
VLDLR.....	Very Low Density Lipoprotein Receptor
vLGN.....	Ventral Lateral Geniculate Nucleus

ABSTRACT

NOVEL ROLES FOR REELIN IN RETINOGENICULATE TARGETING

By Cheryl Virginia Haner RN, B.S.N.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University

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In the developing visual system, the axon of a pre-synaptic cell must be guided to a post-synaptic partner. Retinal ganglion cells (RGCs) in the eye are an excellent model to study this process. Multiple classes exist that respond to specific types of light input, and these project to different destinations in the brain that process distinct types of information. The RGC axons that navigate to the lateral geniculate nucleus (LGN) do so in a class-specific manner. Axons from RGCs that mediate non-image forming functions innervate the ventral LGN (vLGN) and the intergeniculate leaflet (IGL). Axons from RGCs that process image-forming information bypass these regions to innervate the dorsal LGN (dLGN). The extracellular protein reelin was identified as a potential factor in RGC axonal targeting of the vLGN and IGL, and the *reeler* mutant mouse used to study the effects of its functional absence. Anterograde labeling of RGCs and their axons with Cholera toxin B (CTB) revealed reduced patterns of retinal innervation to the vLGN and IGL in mutant mice. Moreover, the absence of functional reelin resulted in axons incorrectly growing into inappropriate regions of the thalamus. We identified these misrouted axons as those of the intrinsically photosensitive RGCs (ipRGCs), a class of

RGCs known to project to the affected subnuclei. In contrast to defects in ipRGC targeting, no deficits were seen in retinogeniculate or corticothalamic projections in classes of axons that normally target the dLGN. Immunohistochemistry did not reveal any effects of the absence of the functional reelin on the LGN cytoarchitecture, which is unlike many other brain regions altered in the *reeler*. In summary, results suggest that intact reelin is required for class-specific retinogeniculate targeting to the vLGN and IGL. The defects are likely to be in targeting and not in neuronal positioning.

INTRODUCTION

As the central nervous system (CNS) develops, the communication of appropriate pre and post-synaptic cells must be established with a high degree of specificity in order to ensure proper functioning. While this concept seems basic, the mechanisms that guide synapse formation are complex and far from entirely understood. The first step in synapse formation is the correct targeting of presynaptic axons to appropriate target cells. Once this axon reaches the appropriate region, it must transform from a mobile, growing structure to a stable bouton capable of releasing neurotransmitters. This step is termed synaptic differentiation. The pre-synaptic axon and the post-synaptic dendrite form the junction that allows for signal transmission between the two cells. Finally, this immature synapse is stabilized if it is to become functional, or withdraws if the incorrect target has been selected (Fox and Umemori, 2006) (Figure 1).

For the purpose of this thesis, only synaptic targeting will be detailed. Early in development, the decisions made concerning the direction of axonal extension are dictated by extracellular cues (Huberman et al, 2008). These ligands exist in the extracellular matrix (ECM) or are membrane bound, and interact with receptors on the growth cone surface. This initiates intracellular pathways responsible for the rearrangement of the cytoskeleton that commits to a directional determination (Huber et al, 2003; Thanos and Mey, 2001). Each of the guided steps taken by a traveling axon, however simple it may seem, involves a balance of various attractive and repulsive cues that send the growth cone onward in the correct direction (Chen and Cheng, 2009). The

extracellular matrix guides axons to general areas, then transmembrane adhesion molecules guide them to the correct cell. In some cases, even subcellular localization is observed and an axon will need to select for a portion of the post-synaptic cell body or processes (Sanes and Yamagata, 2009). Each decision in the progression of a developing axon is critical to the functioning of the organism and must be carefully regulated. A wrong choice made at any of these points can lead to disordered synapse formation and, ultimately, to nervous system dysfunction.

The visual system has received considerable attention in terms of synaptic targeting for a host of reasons. The eye is easily accessible for study and manipulation by the researcher. Retinal ganglion cells (RGCs) project axons from the retina of the eye to many targets in different regions of the brain, making them an excellent model for studying these axon's courses. At multiple junctions en route to a target, a synapse between RGCs and central target neurons will have to respond to positive or negative cues in order to select a direction. In most of these projections, a retinotopic map is maintained so that the location of an RGC synapse within a central visual structure corresponds spatially to its initial location in the retina and within the visual field (Clandinin and Feldheim, 2009). This same map persists in further projections of the post-synaptic cells, such as the primary visual cortex, illustrating the need for precise control of guidance at all stages. Additionally, eye specific domains are found in visual nuclei, demonstrating that the left and right eye inputs must be segregated and guided to certain targets (Huberman, 2007).

In addition to eye specific and topographic mapping, it is now clear that morphologically different classes of RGCs target specific retinorecipient nuclei (Kim et al, 2008). The overall goal of this thesis is to better understand class-specific RGC targeting to retinorecipient nuclei within the brain. As such, it is necessary to review the mechanics of the system and its development.

Path of retinal input

The outside signal that initiates communication of information in the visual system is light, which enters the eye through the cornea (Figure 2A). The retina lies along the back of the inside of eye and is a multi-layered structure housing various types and classes of neurons, each with distinct functions (Figure 2B). Photoreceptors are the primary sensory receptor cells of the retina and are directly stimulated by light, which alters their membrane potential (Figure 3A). Bipolar cells (Figure 3B) relay photic information to the RGCs (Figure 3E) which transmit all retinal input to the CNS. RGC axons can themselves target over 20 distinct CNS regions, each with a different function in the visual system circuitry. Additional types of neurons within the retina, the amacrine and horizontal cells (Figure 3C,D), modulate information conveyed through the above described three neuron circuit (Kandel et al, 2000).

Examining the path of light input through the layered retinal cells helps to demonstrate how specificity must be conveyed at each level of visual processing. When light reaches the retina, it first activates a rod or a cone, the two classes of photoreceptors. The bodies of these cells make up an entire retinal layer toward the back of the eye. Both rods and

cones share similar structure and function, but are stimulated by different inputs. Rods are extremely sensitive, sometimes activated by a single photon, and act by amplifying dim light during night vision. There are three types of cones, each specific to a portion of the light spectrum, and are used for daytime and color vision. The outer segments of both contain membranous discs that are continually shed and replaced, these discs are responsible for phototransduction. The inner segments contain the nuclei and cellular organelles. Internal to those segments are the synaptic terminals, which relay signals from the photoreceptors to bipolar cells (Sanes and Zipursky, 2010).

Within the membranes of the discs, located in the outer segments of both rods and cones, are photosensitive visual pigments called opsins that undergo a conformational change when stimulated by light. Once activated, a component of these membrane-bound pigments shifts from cis to trans. This initiates the intracellular pathway that controls gated ion channels and transmits a signal to interneurons at the synaptic terminal of the photoreceptor. Types of visual information, which will be processed as such in central structures, are first sorted when photoreceptors activate distinct types of bipolar cells, of which there are more than ten. Here, information will be organized by differences such as the onset or offset of light stimulation. The projections of activated photoreceptors, and their downstream post synaptic partners, will convey this class specificity via their targeting of functionally distinct visual nuclei (Kandel et al, 2000; Nolte, 2009).

Melanopsin, another light sensitive opsin, is expressed by a small subset of RGCs; making this a third type of photoreceptor, but the only photopigment, that is present outside of the outer nuclear layer (ONL). Information conveyed by these melanopsin-

expressing cells, however, is quite different from that which stimulates the other photoreceptors and does not project to the same locations (Hattar et al, 2002). This class will be discussed in more detail below.

Internal to the photoreceptor layer, bipolar and horizontal cell bodies in the inner nuclear layer receive input from rods and cones. Bipolar cells project toward more internal layers while horizontal cells facilitate communication between all cell types at this level only. Bipolar cells also articulate with amacrine cells. Both of those cell types form the last set of synapses in the retina, with the RGCs in the inner plexiform layer that will convey visual information out of the eye. These projections will eventually reach the various targets for visual processing within the brain (Sanes and Zipursky, 2010; Kandel et al, 2000).

There are multiple classes of RGCs, identified by morphology and function (Rockhill et al, 2002). These display variations in cell body shape, arborization and stratification that can be used to classify them. Each is specific to its own function of the light processing system, as evidenced by the fact that each class projects to dedicated and functionally distinct locations in the central nervous system. Input that will be recognized as an image comes through the photoreceptors and bipolar cells to exclusively image-forming classes of RGCs. These cells project to subcortical structures that communicate with the primary visual cortex for processing (Yamada et al, 1996). There are different types of image forming classes of RGCs. Direction selective RGCs display a preference for a specific movement; that is, horizontal or vertical movement, of an observed object (Demb, 2007;

Kim et al, 2008). Distinct classes of image forming RGCs are exclusively activated by either increases in light (ON cells) or decreases (OFF cells) (Chalupa and Günhan, 2004; Kim et al, 2008). In order to ensure that light entering the eye is properly sorted and processed, the RGC's axon guidance and synapse formation with the post synaptic cell must be precisely controlled and accurate.

To begin their journey to retinorecipient nuclei within the brain, axons of RGCs travel through the nerve fiber layer of the retina, where growth from all regions of the retina is directed centrally to the optic disc. Here, they form the optic nerve head, which is where all visual information leaves the retina (Figure 2C,D). These projections enter and extend along the optic nerve toward the optic chiasm (Kandel et al, 2000). Eye specific sorting begins to occur here, as fibers from the nasal half of the retina will cross to project to the contralateral side of the brain (So et al, 1990). After passing the optic chiasm, RGC axons enter the optic tract where they travel to distinct central visual nuclei within the brain. Even once they find their appropriate target nuclei, they must be sorted into topographic domains, eye specific domains and class-specific domains (Huberman et al, 2008).

Retinal ganglion cell targets

After crossing in the optic chiasm, RGC axons target many different nuclei in the brain. A major target of these axons is the superior colliculus (SC). The SC receives retinotopically mapped retinal inputs from classes of RGCs and has afferent and efferent connections with multiple CNS pathways. Retinal axons also innervate the

suprachiasmatic nucleus (SCN) of the hypothalamus and the olivary pretectal nucleus (OPN). These nuclei regulate the body's circadian rhythm functions and the pupillary light reflex response, respectively (Nolte, 2009). In addition to these nuclei, retinal innervation occurs in the lateral geniculate nucleus (LGN) in the thalamus, which is of particular interest to us.

In rodents, The LGN acts as a relay center for both image forming sensory input, destined to be processed in the cortex, and non-image forming visual inputs. In mice, roughly 30% of RGCs project to the LGN (Figure 4). Retinal axons that synapse here do so in a retinotopic map along the rostro-caudal axis. Eye specific patterning is also present in the mouse LGN, with contralateral input making up the majority of retinal (Jaubert-Miazza et al, 2005). The LGN can be subdivided into functionally distinct subnuclei, the ventral and dorsal LGN (vLGN and dLGN) and the intergeniculate leaflet, that each receive projections from functionally distinct classes of RGCs. The dLGN primarily processes image forming information and projects to the cortex for conscious recognition (Figure 4A). The vLGN and IGL participate in visuomotor responses and circadian rhythm, both non-image forming processes, and share reciprocal connections with many other visual targets in the brain that the dLGN does not communicate with (Figure 4B,C) (Harrington, 1997).

Although there are many types of morphologically and functionally distinct RGCs, projections of only a few have been identified and warrant attention here. One is the melanopsin expressing, intrinsically photosensitive RGC (ipRGC). Within this class,

there are distinct types sorted by their morphology within the retina. M1 cells have large dendritic arbors with extensive branching located within the OFF sublayer at the outer margin of the inner plexiform layer (IPL). M2 cells have a larger soma and dendritic field than the M1, with monostratified arborization in the inner IPL OFF sublayer. The arbors of this second class are more branched, but the two do overlap. Both of these cell types are intrinsically photosensitive and immunoreactive to staining for melanopsin. Other ganglion cells have shown to have weaker melanopsin immunoreactivity (Berson et al, 2010; Ecker et al, 2010).

The primary targets of these cells are the SCN, the OPN and the LGN. The projections to the LGN mostly synapse in the IGL and the vLGN (Figure 4E), which do not project to the cortex but do share reciprocal connections with the SCN and OPN as part of the non-image forming visual system. There are limited projections from the ipRGC's to the dorsal LGN (dLGN), which does relay information to the cortex and participate in image formation. (Hattar et al, 2002 and 2006) Almost all of these melanopsin expressing cells synapse with nuclei that participate in only non-image forming vision. The link between form and function seen in these cells is typical of class specific segregation in the developing nervous system.

In contrast to the melanopsin-expressing RGCs, calretinin expressing RGCs make up another set of functionally distinct RGC classes in the retina. At least 10 morphologically different classes of image forming RGCs express Calretinin (Lee et al, 2010). While they may be activated by different visual pathways, all of these bypass the vLGN and IGL to

project solely to the dLGN (Figure 4D). However, the different calretinin expressing RGCs seek certain regions of the dLGN upon arrival and the synapses of each class localize in these regions (Lüth et al, 1993).

Guidance of retinal ganglion cells to specific central nervous system targets

Retinal ganglion cells must make crucial directional decisions at various junctions during development in order to synapse in the proper location for their functions. As researchers sought to discover how these choices were made, one initial theory was that RGCs go to all potential targets and rely on experience cues from visual stimulation to refine the arbors formed. Another was that signals exist within the CNS to direct their targeting at the onset. A host of research supports the theory that both of these are required to properly wire the visual system in development (for review, see Huberman et al, 2008). Retinotopic mapping to the SC and eye-specific projections to the LGN, both by RGC axons, occur prior to visual experience (Haupt and Huber, 2008; Huberman et al, 2008). This indicates that an organism's CNS inherently knows how to form circuitry and an intrinsic mechanism of guidance must exist during development. However, a critical period exists after initial mapping that refines synapses in an activity dependent manner (for an example of this, see Hensch, 2005). The effect of experience on RGC synaptogenesis will not be further discussed. However, the identification of some of the major molecular cues in visual patterning will be reviewed.

The first theory proposed to support the influence of inherent molecular guidance cues on axonal migration was Sperry's chemospecificity hypothesis, using retinotectal projections

(Sperry, 1963). The frog optic nerve was severed, the eye rotated 180° and regeneration allowed to occur (it is important to note that this regenerative potential in amphibians does not exist in all species) (Sperry, 1943). When a fly was in the lower visual field, the frog jumped to reach it. A fly overhead caused the frog to strike the ground with its tongue. These mistakes were consistent and never corrected during development. Sperry used these results to suggest that projections from the retinal ganglion cells returned to their original intended targets despite receiving input from a different aspect of the visual field. This was the first argument for an intrinsic axon guidance system that functioned independent of sensory activity (Kandel et al, 2000). Since Sperry's experiments, researchers have tackled decision points in RGC growth cone migration individually, as each involves a different set of extracellular ligands and intracellular response. Some of these interactions and their participants are well understood, while many have yet to be discovered. In order to demonstrate the importance of the varied pool of guidance molecules that an RGC responds to, a few of the better known will be described.

Within the retina itself, RGCs must first differentiate and project axons to the optic fiber layer. It has been determined that Brn3a, Brn3b and Brn3c, expressed by the RGC at this developmental point, play different roles in RGC differentiation and axonal guidance. The guidance of RGC axons in double knockouts of 3b and 3c was more severely affected than that of a single knockout, suggesting the existence of a compensatory role of one in the absence of the other (Wang et al, 2002; Gan et al, 1999). Other studies have found that all three promote the differentiation and migration of RGCs (Liu et al, 2000) in the developing visual system.

Once in the optic fiber layer, secreted Slits repel Robo receptors on the axon to guide it toward the optic disc (Plachez et al, 2008). A retinal dorsal-ventral gradient exists in Slit expression. Double knockouts of Slit1 and 2, or of Slit 2 alone, show a lack of the wild-type's restriction of axons to the optic fiber layer. Single knockouts of Slit 1 show no altered phenotype in terms of layer restriction (Thompson et al, 2009). This again points to a well controlled system of secreted guidance molecules that is required to herd extending ganglion cell axons out of the eye. The same Slit1 and Slit2 molecules are expressed in telencephalon and diencephalon, guiding the same cell projections long after they leave the eye (Thompson et al, 2006). Gradual expression of chondroitin sulphate proteoglycan (CSPG) in the retina corrals these axons toward the optic disc, and incorrect navigation occurs in its absence (Brittis et al, 1992). Secreted sonic hedgehog (Shh) exists in a gradient in the retina, high centrally and low peripherally, and is also necessary for axon extension toward the optic disc. Different expression levels can either have a positive effect (low concentrations) or negative effect on this guidance. Over expression or inhibition of Shh results in a loss of correct disc targeting by the RGCs (Kolpak et al, 2005).

After collecting and exiting the eye via the developing optic stalk, RGC axons extend toward the optic chiasm. Sema5A ensheaths retinal axons to prevent exit from the optic nerve (Oster et al, 2003). Shh appears again at the chiasm. Boc, one of its receptors, is present on ipsilateral RGCs. The interaction of Boc and Shh restricts ipsilateral retinal projections from crossing at the chiasm (Fabre et al, 2010). Additionally, EphrinB2,

expressed at the chiasm by radial glia, interacts with EphB1 on the ipsilateral RGCs to ensure correct guidance at this juncture (Petros et al, 2009). Less is known about the molecules responsible for the crossing of contralateral projections at the chiasm (Garcia-Frigola et al, 2008). It has been demonstrated that this is not a passive process and that it involves a mix of cues (Erskine and Herrera, 2007).

More guidance is required to get axons from the chiasm to their visual nuclei targets. EphA, a receptor tyrosine kinase, is expressed by RGCs. Its ligand, ephrin-A, is present in the LGN. The expression of both of these exists in a central-peripheral gradient, and the repulsion that occurs when they interact has been shown to instruct eye specific patterning in retinogeniculate mapping (Pfeiffenberger et al, 2005). This mapping can be altered by changes in normal visual activity, indicating that both intrinsic signaling and extrinsic stimulation work together to direct synapse formation between RGCs and the LGN (Huberman et al, 2005).

Preliminary identification of retinogeniculate guidance cues

While much is known about retinal axons exiting the eye, crossing at the chiasm, retinotopic maps in the SC and the Eph-ephrin role in the LGN. Little is known about what guides class-specific RGC targeting. In order to identify cues responsible for early navigation of axons from the retina to LGN subnuclei, differential expression of mRNA at the age where this occurs was analyzed. Microarray analysis of the vLGN, IGL and dLGN of wild type postnatal day 3 (P3) brains was used to identify the relative presence of all molecules in each subnucleus (Figure 5A).

In order to further narrow the hunt for a guidance cue specific to early targeting, the timeline of mouse retinogeniculate projection migration must be considered (Figure 6). This begins before birth, and ceases around P14 when the adult pattern has been established. While the initial targeting stage is complete in the first few perinatal days, some classes of RGC axons may continue to target the LGN into the end of the first week. However, refinement of established synapses begins at about P4. After targeting, spontaneous retinal activity begins to refine established RGC arbors (Godement et al, 1984). Molecules that showed an increased mRNA expression in the vLGN and IGL versus the dLGN in the microarray were examined in the brains of progressively aging perinatal and postnatal mice. Quantitative Polymerase Chain Reaction (QPCR) analysis identified *reln* as one cue that is highly enriched in vLGN/IGL at P3, but its expression tapers steadily as retinal axons are no longer entering the LGN (Figure 5B).

Reelin is known to act in axon guidance in other regions of the brain. We found its mRNA to be enriched in the vLGN and IGL, both recipients of projections of non-image forming RGCs. This expression coincides with targeting of these RGCS to the LGN. For these reasons, we focus the studies in this thesis on the role of reelin in retinogeniculate targeting.

Reelin

The spontaneously generated *reeler* mutant was identified over 50 years ago, and has been used as a model to study the causes and effects of defective brain cytoarchitecture (Falconer, 1951). It results from a mutation in the *Reln* gene (D'Arcangelo et al, 1995). A

protein is still produced, but it does not function properly as it does in the wild type mouse. Phenotypically, the mutant is smaller than its littermates and displays an ataxic gait and tremor (Fatemi, 2008). The layered structures in the brain, including the cerebral and cerebellar cortices and the hippocampus, show disorganization of cells (Frotscher et al, 2009). Human psychiatric disorders that may involve disruptions in this gene include schizophrenia, autism and attention deficit disorder (Knuesel, 2010).

Reln codes for a glycoprotein that is secreted throughout development and adulthood. This protein initiates a cascade via its interaction with membrane bound receptors, apolipoprotein Receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR). Once bound, these ligands form homodimers that bind and phosphorylate Dab1, an intracellular adapter protein. Downstream effects of this phosphorylation include modulation of tau and the actin cytoskeleton, which control migration of neurons. (Figure 7) (Katsuyama and Terashima, 2009). A similar mutant phenotype is displayed by animals with mutations in the *dab1* gene, which codes for Dab1 (Howell et al, 1997), and in mice that have both VLDLR and ApoER2 knocked out (Trommsdorff et al, 1999).

Reelin is known to contribute to the formation and retinal targeting of one retinorecipient nucleus, the SC. Reelin is expressed in the uppermost layers of the developing SC, prior to arrival of RGC axons. Retinotectal targeting is perturbed in the absence of reelin, however the lamination of the SC is also disrupted in *reeler* mutants, a defect that occurs prior to retinotectal targeting (Baba et al, 2007). For this reason, it has been proposed that retinal targeting defects are secondary to lamination defects.

The effects of reelin on the LGN have not been studied. This thesis seeks to identify possible roles for reelin in retinogeniculate targeting. Preliminary studies found its mRNA expression increased in the vLGN and IGL in microarray analysis of the P3 wild type, with a decrease in the presence of the protein as retinogeniculate migration slows with development. These findings were the foundation for further examination of the *reeler* mutant to identify its role in guidance of RGCs to the mouse LGN, focusing on class specific targeting to its subnuclei.

Figure 1 Synapse Formation

In the first step of synapse formation, a pre-synaptic axon extends toward a post-synaptic target **(A)**. Once it arrives, it differentiates, making it a functional synapse able to transmit information via the release of neurotransmitters. **(B)**. After an excess of immature synapses form, each must either be stabilized and maintained or eliminated **(C)**.

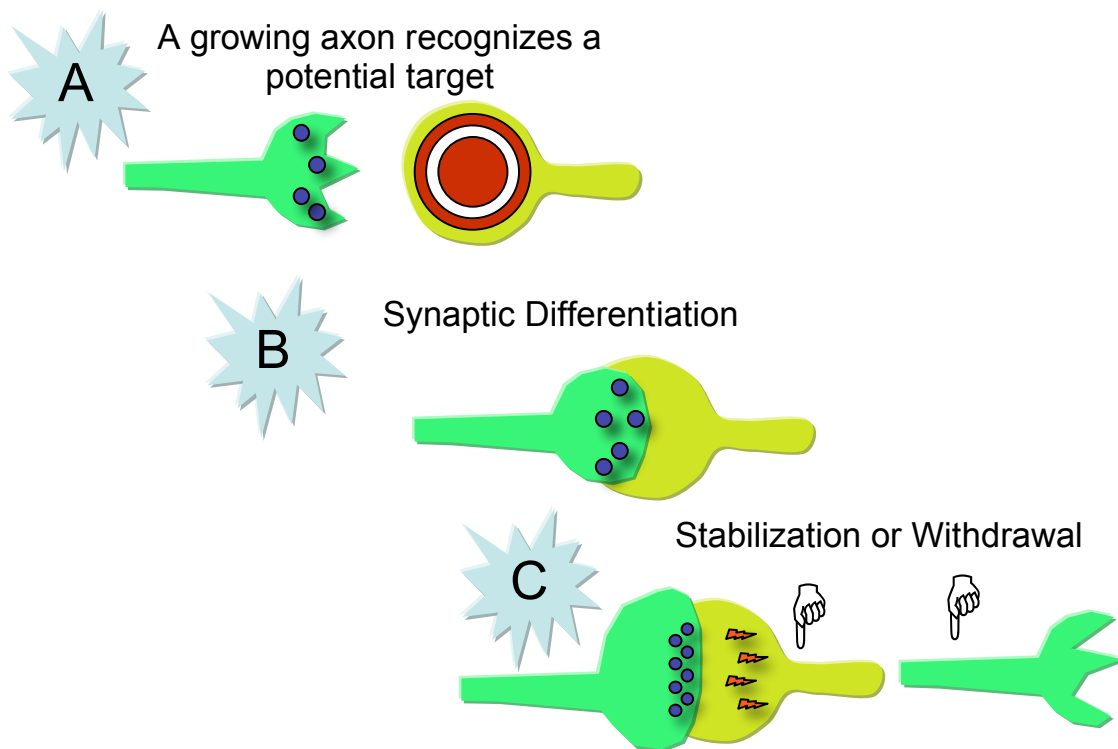


Figure 1 Synapse Formation

Figure 2 Eye

Visual input, in the form of light, enters the eye through the cornea **(A)** and travels to the through the retina **(B)** to its innermost layer. From here it travels through layers of retinal cells to collect in the optic disc **(C)**. All information leaving the eye does so via the optic nerve **(D)**.

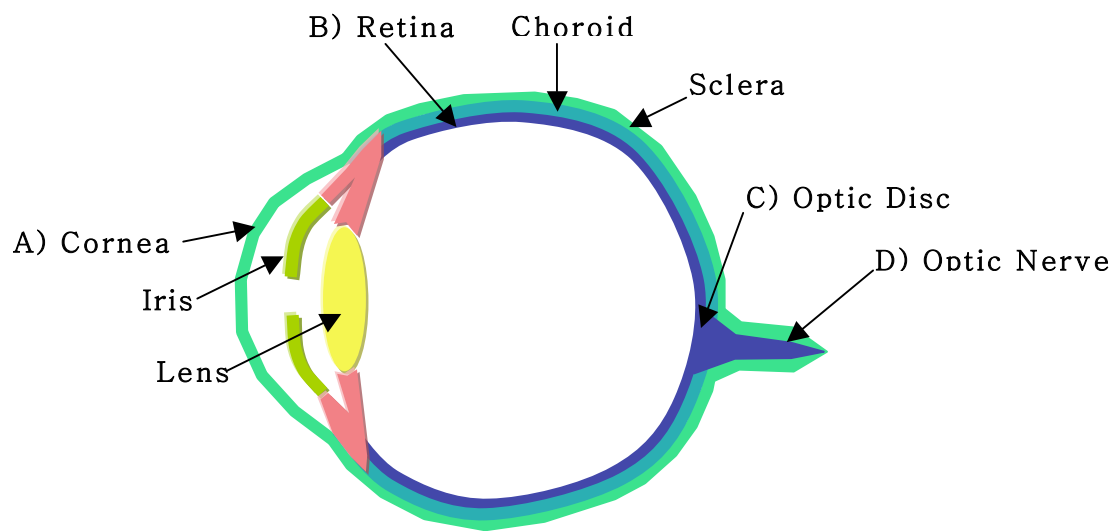


Figure 2 Eye

Figure 3 Retinal Cell Types

Photoreceptors **(A)** are the recipients of incoming light and convert it to nervous information to convey to the brain. Horizontal cells **(B)** modulate the photoreceptor synapses. Bipolar cells **(C)** are activated by photoreceptors and relay such activity to RGCs. Bipolar and Amacrine cells **(D)** are present in the INL. Retinal Ganglion Cells **(E)** are the projection neurons of the retina and project axons to the optic nerve to innervate retinorecipient nuclei in the brain.

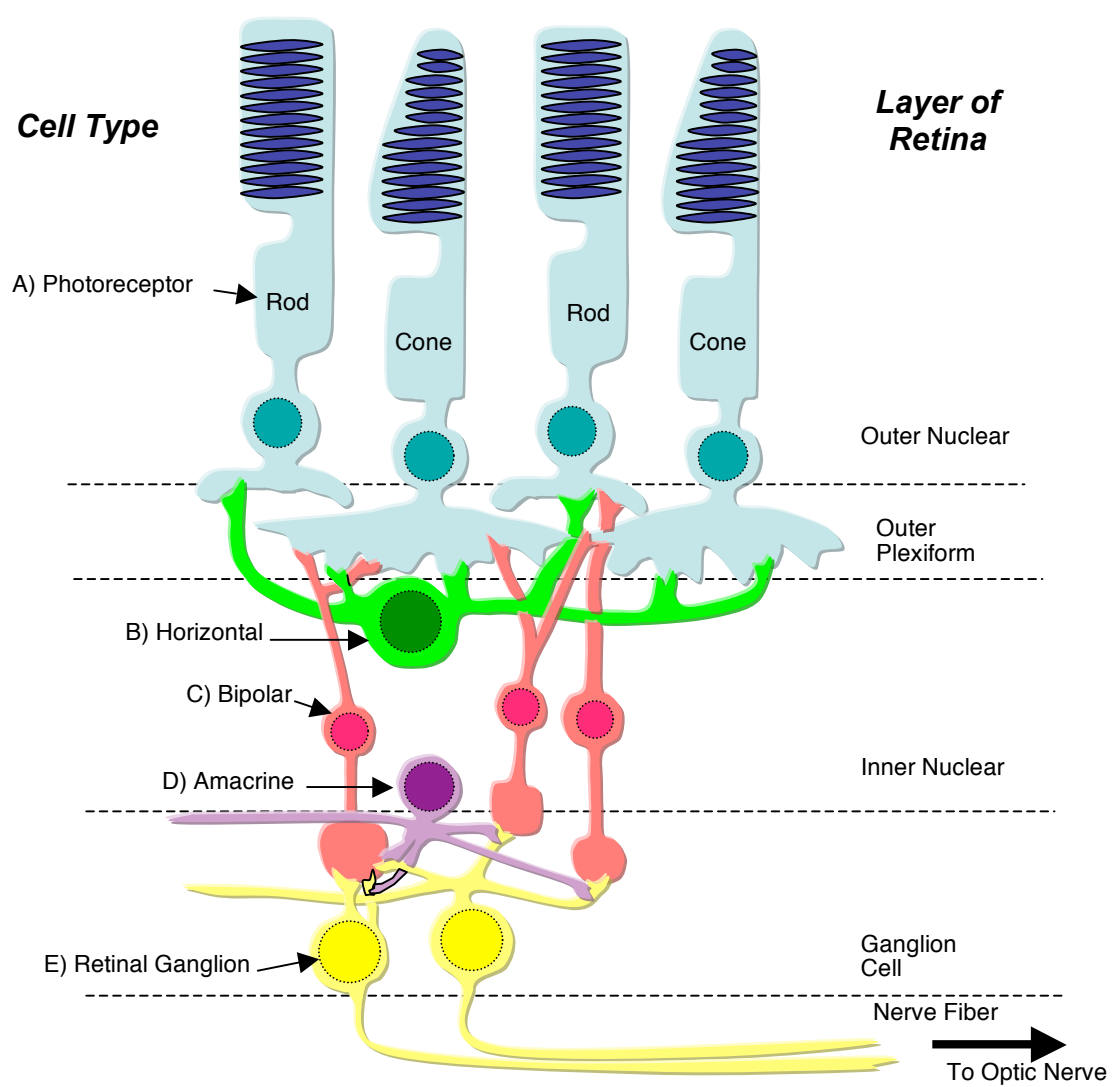


Figure 3 Retinal Cell Types

Figure 4 Retinogeniculate Projections

(I)

The dorsal LGN (**A**) receives image forming input from the retina and projects it to the primary visual cortex. The ventral LGN (**B**) and the intergeniculate leaflet (**C**) receive non-image forming retinal input and share reciprocal connections with other non-image forming recipients of RGC projections.

(II)

Image forming classes of RGCs (**D**) bypass the vLGN and IGL to form arbors in the dLGN. Non-image forming classes of RGCs (**E**) carry visual information to the vLGN and IGL only.

LGN= Lateral Geniculate Nucleus, dLGN= dorsal LGN, IGL= Intergeniculate Nucleus,
vLGN= ventral LGN, OT= Optic Tract, ON= Optic Nerve, OC= Optic Chiasm

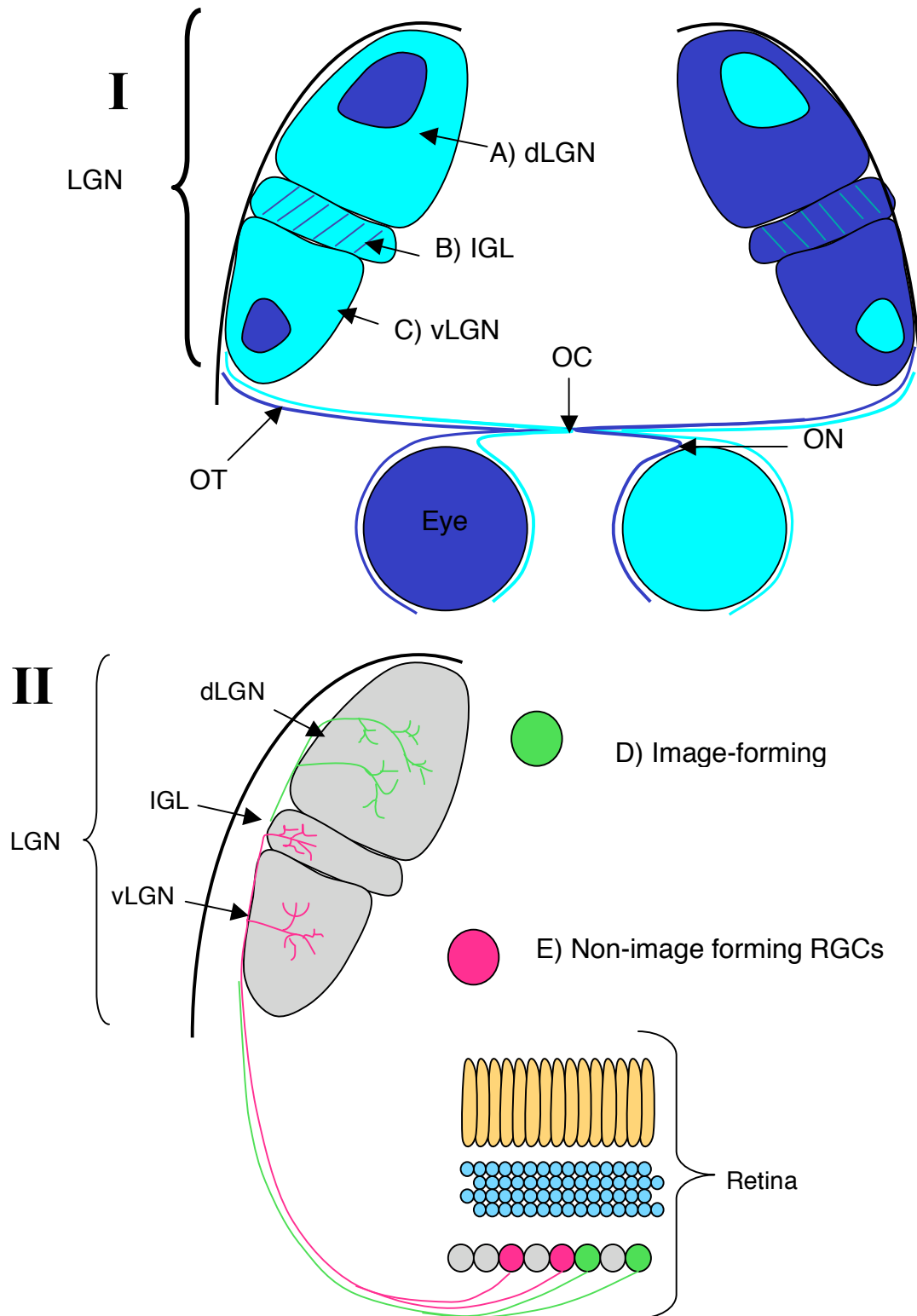


Figure 4 Retinogeniculate Projections

Figure 5 Differential expression of ECM genes in the LGN subnuclei

(A)

Microarray analysis of P3 LGN tissue was performed to identify extracellular guidance cues that showed different levels of expression between nuclei.

(B)

Extracellular molecules that showed a higher level of expression in the vLGN and IGL were further examined using qPCR at various ages through development **(B)**.

Reln showed an enrichment pattern that indicated a potential role in early axonal guidance, decreasing as the animal aged.

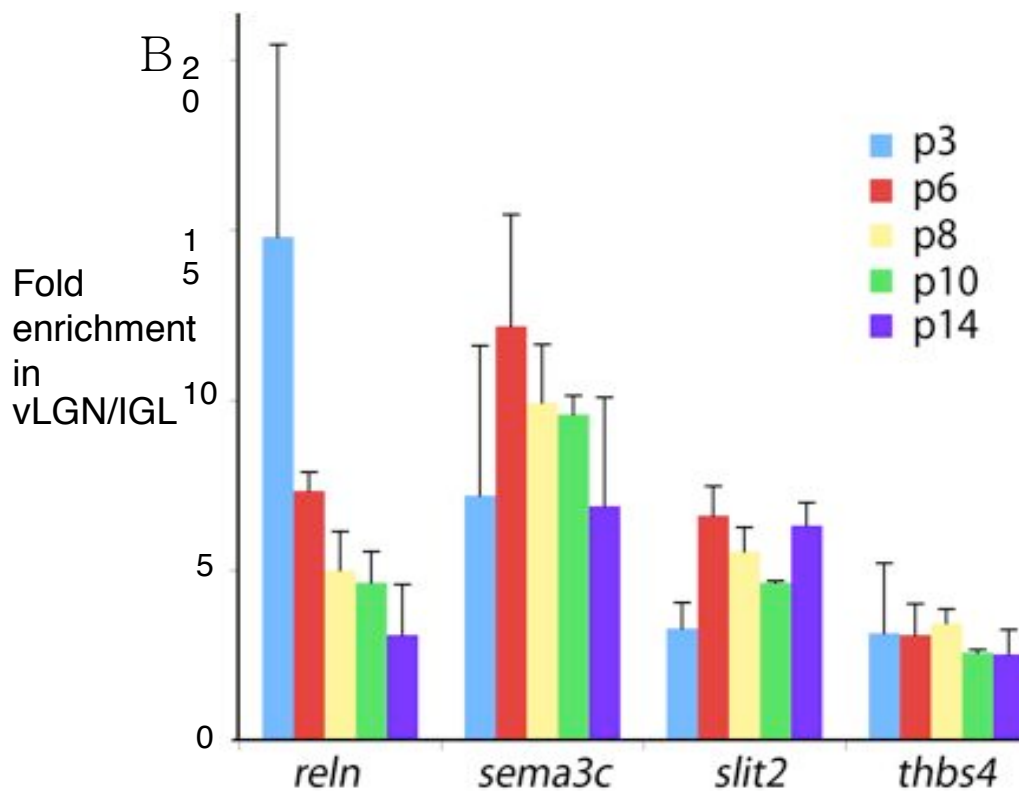
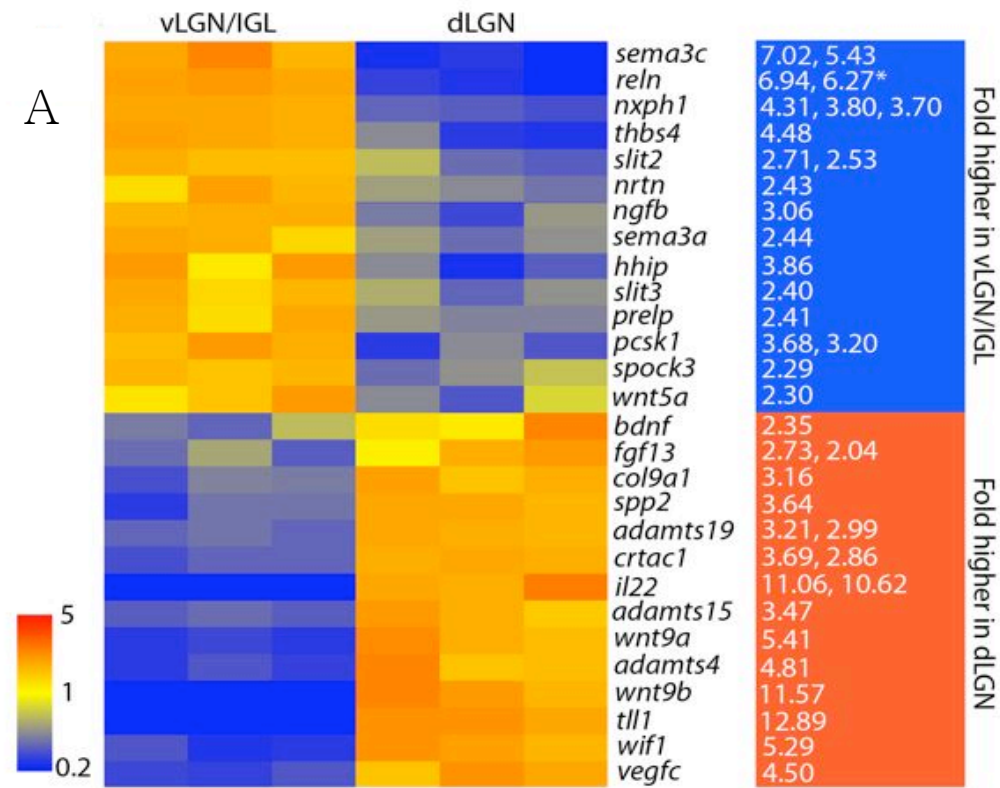


Figure 5 Differential expression of ECM genes in the LGN subnuclei

Figure 6 Timeline of Retinogeniculate Development

From E18 to P8, retinal axons arrive and arborize in the LGN (**A**). At roughly P4, binocular competition begins pruning retinal arbors into eye-specific domains (**B**). From P8 until P14, when the adult pattern of synapses is established, refinement and visual experience drive retinogeniculate development (**C**).

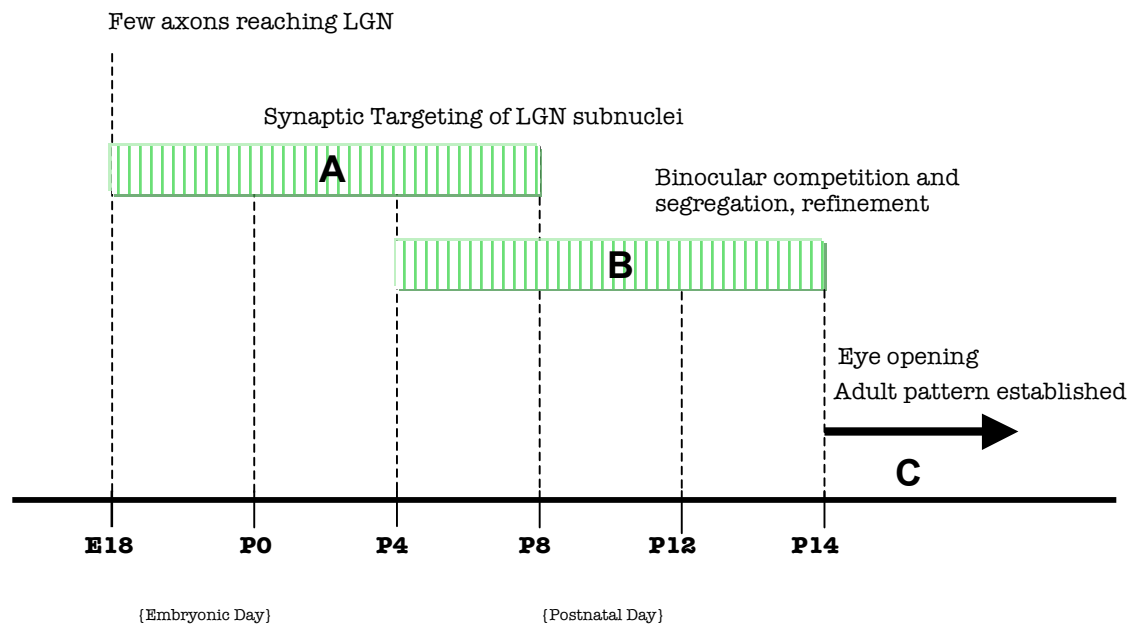
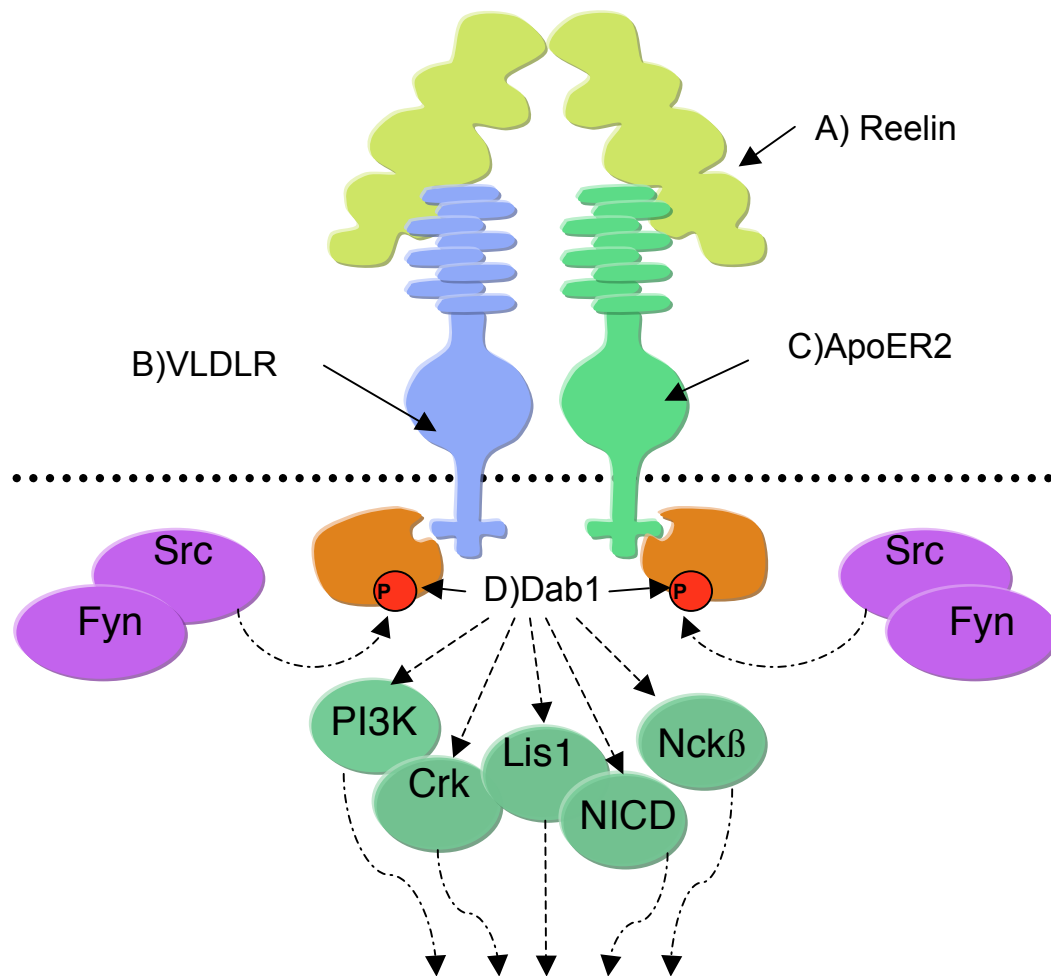


Figure 6 Timeline of Retinogeniculate Development

Figure 7 Reelin Pathway

Secreted reelin glycoprotein (**A**) interacts with membrane bound receptors VLDLR (**B**) and ApoER2 (**C**). Once bound, these form homodimers that bind and phosphorylate Dab1 (**D**), an intracellular signaling molecule. This can initiate multiple pathways that ultimately lead to downstream effects on the migration of axons via cytoskeletal arrangement.



Effects on Neuronal Migration Via Cytoskeletal Arrangement

Figure 7 Reelin Pathway

MATERIALS AND METHODS

1) Reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless otherwise noted.

2) Antibodies

All primary antibodies applied in these studies are described in *table 1*. All fluorescently conjugated secondary antibodies were purchased through Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) or Invitrogen (Carlsbad, CA) and were applied at a 1:1000 dilution. CTB was from Invitrogen (table 1).

3) Animals

The following mice were used in the studies described in this thesis. Wild-type C57 and CD1 mice were obtained from Charles River Inc. *Reln*^{+/-} heterozygote mice were obtained from Jackson labs and a colony was generated. *VLDLR*^{-/-} mutants, aged roughly postnatal day 30 (p30), were obtained from Jackson labs and immediately used in these studies. *Dab*^{+/*scm*} and *Dab*^{*scm/scm*} were obtained from Jackson labs. Golli-tau-GFP mice, which specifically labeled layer VI cortical neurons with GFP (Jacobs et al, 2007), were obtained from a collaborator (Dr. William Guido, VCU) and were crossed onto the *Reln*^{+/-} background. *Opn4*-tau-LacZ mice, in which M1 intrinsically photosensitive retinal ganglion cells (ipRGCs) are labeled with LacZ were obtained from Dr S. Hattar (Johns Hopkins University). All analyses conformed to National Institutes of Health

Antigen	Isotype	Description of immunogen	Source/catalog number	Dilution for IHC
Calbindin	Rabbit polyclonal	Rat Calbindin D-28k	Swant #CB-38a	1:1000
Calbindin	Mouse IgG ₁	Purified chicken gut calbindin D-28k	Swant #300	1:1000
Calretinin	Rabbit IgG	Recombinant rat calretinin	Millipore #AB5054	1:2000
Disabled 1 (Dab1)	Rabbit Polyclonal	Synthetic peptide corresponding to amino acids 400-455 of mouse Dab1	Abnova #PAB10198	1:2000
Glial fibrillary acidic protein (GFAP)	Rabbit Polyclonal	GFAP isolated from cow spinal cord	DakoCytomation #Z0334	1:1000
Glutamate Decarboxylase 67 (GAD67)	Mouse IgG _{2a}	Recombinant GAD67 protein	Chemicon #MAB5406	1:1000
Glutamate Decarboxylase 65 & 67 (GAD 65/67)	Rabbit polyclonal	Synthetic peptide containing a Cysteine residue and amino acids 572-585 of rat GAD65	Chemicon #AB1511	1:500
Green Fluorescent Protein	Rabbit polyclonal	GFP purified directly from the jellyfish <i>Aequorea victoria</i>	Invitrogen #A-6455	1:250
Melanopsin (VCU013)	Rabbit polyclonal	N-terminal most 15 amino acids of mouse melanopsin	CK Chen, VCU	1:1000 (whole mount retina), 1:2000(sectioned retina)
Neurofilament H Non-phosphorylated (SMI32)	Mouse IgG ₁	Ascites fluid	Covance #SMI-32R	1:1000
Neuronal Nuclei (NeuN)	Mouse IgG ₁	Purified cell nuclei from mouse brain	Chemicon #MAB377	1:100
Neuropeptide Y Antibody (NPY)	Rabbit polyclonal	Neuropeptide Y coupled to bovine thyroglobulin	Immunostar #22940	1:250
Synaptic vesicles (SV2)	Mouse IgG1			1:50
Synaptotagmin 2 (ZNP1)	Mouse IgG _{2a}	1-5 day zebrafish embryo	Zebrafish International Resource Center	1:250
Vesicular Glutamate Transmitter 1 (VGLUT1)	Mouse IgG1	Fusion protein amino acids 493-560 of rat VGLut1	NeuroMab # 75-066	1:100
Vesicular Glutamate Transmitter 2 (VGLUT2)	Rabbit polyclonal	Fusion protein of amino acids 510-582 of rat VGLut2	Synaptic Systems #135403	1:500

Table 1: Antibodies

(NIH) guidelines and were carried out under protocols approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee.

4) Genotyping

Tails were cut from mice and dropped into 75 µl of 25 mM NaOH, then incubated at 90°C for 45 minutes. Once removed from heat and cooled on ice for 2 minutes, tails were broken up and 75 µl of 40 mM Tris-HCl was added. After centrifuging, the supernatant was pulled off to perform genotyping. All primers were purchased from Integrated DNA Technologies and an Eppendorf Mastercycler EP was used for all PCR. Genotyping for wildtype reln DNA was performed with 5'-TTA ATC TGT CCT CAC TCT GCC CTC T- 3' and 5'-GCA GAC TCT CTT ATT GTC TCT AC-3'. Mutant reln DNA was detected with 5' - TTC CTC TCT TGC ATC CTG TTT TG-3' and 5'-TTA ATC TGT CCT CAC TCT GCC CTC T- 3'. For reln genotyping, the reaction was run at the following settings: 94 °C for two minutes, 30 cycles of amplification (94 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for thirty seconds) 72 °C for 10 minutes then cooled to 4°C. The primers used to identify GFP were 5'- AAG TTC ATC TGC ACC ACC G – 3' and 5'- TCC TTG AAG AAG ATG GTG CG -3'. The program run for GFP identification is 94°C for 1 minute and 30 seconds, thirty five cycles of amplification (94°C for 30 seconds, 55 °C for 1 minutes, 72 °C for 1 minute) then 2 minutes at 72 before cooling to 4 °C. LacZ and Opn4 were detected with 5'- TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA -3' and 5'- ATG TGA GCG AGT AAC AAC CCG TCG GAT TCT -3'. The PCR program used for both was: 95 °C for 5 minutes, thirty five cycles of amplification (95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 30

seconds) followed by 10 minutes at 72 °C before cooling to 4 °C. Due to the nature of the point mutation responsible for the Dab mutant, an accurate primer has not been identified and genotyping was not done. Results were obtained by running through 1.5% Agarose in tris-acetate buffer with EDTA (TAE).

5) Intraocular injections of anterograde tracer

Prior to animal handling; glass pipettes, with or without filaments, were pulled on in the laboratory of Dr William Guido. Mice were anaesthetized with isofluorane vapors. The vitreous was drained under a dissecting scope by piercing the sclera with a glass pipette containing a filament. A second pipette, lacking a filament and instead filled with 1-2% solution of the B subunit of cholera toxin (CTB, Invitrogen) conjugated to AlexaFluor 594 or 488 dissolved in water (with 10% DMSO), was then inserted into the hole vacated by the first pipette. A picospritzer attached to this second pipette injected 2-6 µl of CTB conjugate into the eye. Pulses of CTB were 15 ms in duration with 12-18 psi of pressure, depending on the age of the mouse. In some cases, two different conjugates were used to identify eye specific projections in the LGN. A one to two day survival period following intraocular injection allowed for filling of the LGN by the tracer.

Following this survival period, mice were sacrificed by subcutaneous injection of 150 mg/ml sodium pentobarbital. The sternal plate was removed, the heart exposed and phosphate buffered saline (PBS) was transcardially perfused through the mouse's circulatory system. Following PBS, mice were perfused with >4% paraformaldehyde (PFA) in PBS. After removal and exposure of the brain, the head was post-fixed in 4%PFA solution overnight before washing with PBS several times. Fixed brains were

then blocked in 4% agarose (in PBS) and 80-100 μm thick, coronal sections were cut and serially collected using a Leica VT 1000S vibratome. These sections were floated in PBS until mounted on slides with ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, Oregon).

6) Immunohistochemistry

A) Retina dissection

Following transcardial perfusion with PBS then 4% PFA in PBS, eyes were removed from mice, placed in 4% PFA in PBS and dissected immediately. The sclera was pierced with a 22G needle and 4 cuts were made around the edges of the hole. Opposite corners were tugged with tweezers until the lens could be popped out. The sclera and retina were then carefully separated by pinching the sclera with fine tipped tweezers and sliding the retina off with a second pair. For those retinas intended for whole mount preparation, cuts were made with spring scissors in each of the four quadrants to ensure that the retinas would lay flat once fixed. Cut retinas were floated in PFA for several hours before being stained by IHC or before mounting with either ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, Oregon) or Vectasheild (Vector Laboratories, Burlingame, CA). Alternatively, following fixation, some retinas were cryopreserved in 25% sucrose in PBS overnight. Once these retinae no longer floated in sucrose solution, they were placed in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrence, CA), frozen at -80°C and sectioned at $16\mu\text{m}$ on a Leica CM 1850 cryostat. See IHC below for the staining and imaging of these sections.

B) Brain dissection

Following transcardial perfusion with PBS then 4%PFA in PBS, mouse brains were dissected and post-fixed in 4% PFA in PBS at 4°C for 12 hours. Brains were then washed with PBS and left in 25% sucrose in PBS for a minimum of 24 hours. Once fixed and submerged in sucrose solution, brains were blocked and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrence, CA), then sectioned at 16µm on a Leica CM 1850 cryostat.

C) IHC on sectioned tissues

Sections were air dried for 15 minutes and then incubated with blocking buffer (2.5% normal goat serum, 2.5% bovine serum albumin, 0.1% Triton X-100 in PBS) for 20 minutes. Primary antibodies were diluted in the same blocking buffer at various concentrations. The sections were incubated with these at 4°C for 12 hours. Sections were washed three times for 10 minutes in PBS before incubating in secondary antibodies, diluted in PBS at a 1:1000 concentration, for one hour at room temperature. After another three 10 minutes washes with PBS, slides were mounted with Vectasheild (Vector Laboratories, Burlingame, CA) for imaging. Visualization was done on either a Leice SP2 scanning confocal microscope or a Zeiss Axiolmager A1 fluorescent microscope. Manipulation of fluorescent images was performed using Adobe Photoshop CS3 (San Jose, CA).

D) Whole mount retina staining

Staining of whole mount retinas was done in the same manner as brain sections.

However, primary antibodies were incubated for 48 hours and secondary antibodies for

1-2 hours. PBS washes were extended, for a total wash time of 2 hours after both primary and secondary applications.

7) Cresyl violet stain

Mouse brains were fixed in 4% PFA in PBS for at least 12 hours at 4°C and then washed in PBS several times. 100µm sections were cut on a Leica VT 1000S vibratome, placed on slides and air dried overnight. These slides were then submerged sequentially in 95% and 70% ethanol, then distilled water. Staining with filtered cresyl violet solution (5 mg/ml cresyl violet acetate, 0.34 M acetic acid, 60 mM sodium acetate) was performed for 1-4 minutes. Slides were rinsed in distilled water before dehydration with 70%, 95% and 100% ethanol. After 10 minutes of immersion in HistoClear (National Diagnostics, Atlanta, GA) and air drying, slides were cover-slipped with Permount (Fisher Chemicals, Fair Lawn, NJ).

8) Imaging

Fluorescent images were taken using a Zeiss AxioImager A1 fluorescent scope with an AxioCam MRm. Confocal images were taken with a Leica SP2 scanning microscope. Cresyl Violet staining was imaged with a Nikon ECLIPSE E800M fluorescence microscope equipped with a Diagnostic Instruments Spot RT CCD camera.

9) *LGN measurments*

Patterns of retinal innervation to the LGN subnuclei were measured using AxioCam MRm. The entire LGN was studied by including all obtained sections. 12-18 sections were examined, with the number varying between animals. Six controls and six mutants were used. The outline of projections to the vLGN, IGL and dLGN were measured individually so that their relative size in pixels could be analyzed (Figure 8).

Figure 8: Measurement of Retinogeniculate Projections

Outlines of RGC projections to each LGN subnucleus were manually drawn based on CTB fluorescence. After circling the 3 LGN subnuclei, relative pixel areas were calculated. An example of the area of the dLGN in one CTB labeled coronal section is shown.



Figure 8: Measurement of Retinogeniculate Projections

RESULTS

1) Reelin protein expression is high in the vLGN and IGL

As discussed in the introduction, microarray and qPCR results indicated a higher expression of reelin mRNA in the ventral LGN (vLGN) and intergeniculate leaflet (IGL) than in the dorsal LGN (dLGN). Since mRNA expression levels do not always directly indicate protein levels, we next examined the distribution of reelin protein in the developing LGN. Moreover, we were interested in examining the developmental regulation of reelin, as the expressions of many axonal guidance and targeting cues coincide with the aspect of development that they are important for. To examine differential protein expression, immunohistochemistry (IHC) was performed on the mouse LGN at ages corresponding to the arrival, arborization and refinement of retinal arbors.

We first examined reelin expression at P0, an age in which RGC axons are just reaching and innervating the LGN and are locating the proper target subnuclei (Godemont et al, 1984). Fluorescent IHC on 16µm coronal sections revealed reelin is present and abundant in the vLGN and IGL but is largely absent from the dLGN and other adjacent thalamic nuclei (Figure 9A). Reelin expression in the cortex and hippocampus matched previous reports (Katasuyama and Terashima, 2009). The expression pattern in the LGN could indicate two things. First, based on the stage of development at P0, reelin may act as a molecular guidance cue for retinal axons targeting subnuclei of the LGN. Further, based

on the localization of expression in the vLGN and the IGL, a role in class specificity may be implied.

We next examined reelin expression at P3 and P7 (Figure 9B,C). At these ages, retinal axons are still growing into the LGN, and eye specific patterning and retinotopy are being established (Huberman et al, 2008). We found that reelin protein expression was still enriched in the vLGN and IGL compared with the dLGN, but that expression was reduced compared to P0. It was still seen almost exclusively in the same two subnuclei. This further supports the argument for reelin as a cue used to direct axons that reach the vLGN and IGL. These results also confirm the validity of microarray and qPCR as methods to identify developmentally regulated signaling molecules.

As synaptic refinement begins to occur alongside targeting, it could be assumed that the expression of guidance cues would decrease as less axons are actively navigating toward the LGN. From P7-P10, refinement of established connections is occurring, through activity-dependent processes (Huberman et al, 2008). By P14, when the eyes are open, formation and refinement of retinal ganglion circuits are complete. Reelin IHC on P14 coronal mouse brains (Figure 9D) demonstrated little, if any, extracellular expression in any region of the LGN. However, intracellular expression of reelin was seen in a few individual cells scattered throughout all LGN subnuclei.

In summary, at ages when it is known that intrinsic developmentally regulated signaling molecules are the dominant factors controlling new synapse formation; reelin protein is

expressed in high levels. By the age when RGC axons are no longer entering and arborizing in the LGN, it appears that the expression of the reelin protein decreases as well. Taken together, the results suggest that reelin may play a role in directing axonal targeting to these thalamic nuclei.

2) RGC projections to the vLGN and IGL are diminished in the reeler mutant

After completion of immunohistochemical studies to locate reelin protein expression in the LGN, the next step was to determine the necessity of the protein in its development. The method used to investigate this was a comparison between a mutant lacking functional reelin and a wild type littermate control. The *reeler* mouse has been studied for over fifty years (Falconer, 1951). Its spontaneous mutation results in a presentation of ataxia, tremors and a characteristic reeling gait. These offspring are generally smaller than their littermates and only about 20% survive beyond weaning. Examinations of the cytoarchitecture in the brains of these mutants have revealed a disorganization of the cells in layered structures, such as the cerebral and cerebellar cortices, the superior colliculus and the hippocampus (Frotscher et al, 2009; Sibbe et al, 2009; Badea et al, 2007). The phenotype was found to be the result of a mutation of the *reln* gene, which codes for a secreted protein found in parts of the developing central nervous system (D'Arcangelo et al, 1995). The effects of this altered protein on the organization of the developing LGN have not been previously described. As we had already discovered an abundance of reelin present here in control animals, with differential expression among subnuclei, it seemed worthwhile to look for alterations in this nucleus in the *reeler* mutant.

Cholera toxin B (CTB) intra-ocular injections were used to examine the patterning of RGC arbors in mutant and wild-type LGNs. The B subunit of CTB helps the toxin to be endocytosed by the RGC soma, and it is then transported down the RGCs axon, allowing visualization of its retinal arbors. After allowing animals to survive for one to two days, the projections of these cells to the LGN were visualized and measured.

We first looked at P14 animals. At this age, when the eyes are opening, axonal targeting and refinement are complete (Godement et al, 1994). Eye injections performed in mutants and wild type animals produce clearly visible structural differences at this stage of development. In mutants the retinal projections to the IGL are missing or greatly reduced. Projections to the vLGN also appear decreased, though less dramatically so. Because these two subnuclei are innervated by less RGCs, the relative percentage of total input to the LGN that reaches the mutant dLGN appears much greater than what is seen in controls (Figure 10A,B).

Measurements and comparison of the area occupied by RGC projections to the vLGN, IGL and dLGN were done to further explore specificity in guidance defects here. After CTB injection and sacrifice, 80-100 micron sections were taken from animals and imaged in series. These images were lined up, from rostral to caudal ends and the pixel areas measured for each LGN subnucleus (Figure 12A-C). Measurements were not of the total nucleus, but of the area occupied by RGC projections to each. These values were used to compare the relative pattern of retinal innervation to the vLGN, IGL and dLGN.

Inclusion of the entirety of each region was meant to confirm that any changes observed were not the result of axons projecting to a different location within the same subnucleus.

The alterations observed in the previous images were confirmed by relative area comparison. The control nuclei were adjusted to 100% and the mutant areas measured and compared to these values (Figure 13). Relative to the control, the area occupied by retinogeniculates to the mutant IGL was roughly 45% smaller. Projections to the mutant vLGN were 16% smaller. The RGC axons making it to the dLGN made up 10% more area in the mutant compared to the control.

In addition to the altered nuclei, misrouted axon tracts are seen projecting away from the LGN toward other thalamic nuclei. In control mice, RGC axons terminals remain in the LGN or the optic tract as it courses through the thalamus. *Reeler* mutants displayed axons exiting from the vLGN and at the IGL-dLGN border (Figure 11), but none were seen leaving the dLGN itself. The gross misrouting of retinal axons in *reeler* mutants supports the argument that properly functioning reelin protein is essential for correct retinogeniculate development. The injections of different fluorescently-labeled conjugates of CTB into each eye showed that these misrouted axons arise from RGCs in both ipsilateral and contralateral retinas (data not shown). Based on the diminished retinal innervation of the IGL and vLGN, and the location of misrouted retinal axons, it appeared likely that RGC axons targeting these subnuclei lacked an appropriate cue in *reeler* mutants. Together, this data demonstrates that the functional reelin protein is required for non-image forming retinogeniculate targeting.

During embryonic development and the first few postnatal days, all incoming RGC axons first encounter the vLGN and IGL before projecting to the dLGN. In order to make the initial decision whether to synapse or move on to another potential target, developmentally regulated guidance cues must direct the extending axons. Based on the developmental expression pattern and the defects seen in P14 reeler mutants, it could be assumed that reelin is required to direct the initial targeting of retinal synapses in the vLGN and IGL. However, by P14, RGC targeting into the LGN has ceased and pruning of these retinal arbors is occurring. Therefore, an alternative possibility is that retinogeniculate defects are due to faulty reorganization of retinal arbors in the reeler LGN and not due to initial targeting defects. In order to determine which of these possibilities is correct, younger mice with still active axonal guidance as the dominant force in synaptic specificity were studied.

To distinguish whether reelin was required for the initial targeting, or for subsequent refinement, we examined RGC arbors in the LGN at younger ages. P1 mice were examined first. At this age the driving force of synaptogenesis is target seeking by the presynaptic axon. Injected P1 mutant RGC axons still displayed reduced patterns of innervation to the vLGN and IGL (Figure 14B) and were misrouted away from the IGL into other thalamic nuclei (Figure 14D, D'). The fact that this pattern is established in the absence of functional reelin at this early developmental stage supports a role for reelin in the initial targeting and formation of retinogeniculate circuits. As differences are only

apparent in the two of the three subnuclei, only axons that target the vLGN and IGL require reelin for proper guidance.

In addition to examining retinal axons at P1, we also examined retinogeniculate targeting in P7 mice. The same variations in LGN patterning seen at P14 and P1 were observed in these ages. Although measurements were not taken, the IGL and vLGN again appear smaller in the mutant, with aberrant projections toward other regions of the thalamus not seen in the wild type.

3) ipRGCs are morphologically normal in reeler mutants

Eye injections suggest that the defects present in the *reeler* mutant RGC projections to the LGN are the result of faulty targeting, not refinement. However, an alternative possibility is that these flaws are the result of altered cell development in the retina, as opposed to incorrect guidance in the LGN. Some classes of RGCs are known to participate in only non-image forming visual processing and target the vLGN and IGL without projecting to the dLGN. The case may be that these RGCs are absent or unable to generate axons in the retina. Melanopsin, an opsin like photopigment, is expressed by one subset of RGCs (Provencio et al, 2000). These are directly sensitive to light and bypass the photoreceptors and bipolar cells to project to the SCN, vLGN, IGL and OPN (Hattar et al, 2002). There are no projections from these cells to the dLGN (Figure 15A).

In order to determine whether these RGCs were affected by the faulty reelin protein before leaving the eye, a melanopsin antibody was applied to whole mounted and

sectioned retinas. This antibody, generated by Dr. Jason Chen, was specific to the N-terminal most 15 amino acids. Similar antibodies have been shown to detect all isoforms of melanopsin and therefore all classes of ipRGCs. Whole mounted mutant retinas were compared to the littermate controls (Figure 15B-D). No gross changes in ipRGC morphology were noted. Dendritic arbors in both are highly branched and ipRGC axons in controls and mutants projected toward the optic disc (Figure 15C,D). Thus, ipRGCs appeared morphologically normal in *reeler* mutants. Similar results were observed in retinal cross-sections (Figure 16). In both the mutant and the control animals, melanopsin expressing RGC bodies were located correctly in the ganglion cell layer with dendrites along either border of the inner plexiform layer. Moreover, mutant ipRGCs were equally capable of endocytosis of CTB, therefore defects in retinogeniculate targeting were not the result of faulty labeling in mutant retinas. This confirms that the absence of a functional reelin protein does not affect ipRGCs within the retina.

In addition to assessing ipRGCs within the *reeler* retina, we examined whether ipRGC axons were capable of targeting other appropriate retinorecipient nuclei in mutants. The OPN and SCN also receive projections from ipRGCs and function in the same non-image forming circuits as the vLGN and IGL. In order to determine whether targeting defects in the *reeler* mouse were exclusive to the LGN, these other two nuclei were studied. No irregular routing of retinal axons was seen, confirming limited targeting defects in the absence of the functional reelin protein (Figure 17).

4) ipRGC axon targeting to the vLGN and IGL is disrupted in the reeler mutant

The projections of ipRGCs were studied in the LGN to look for alterations in targeting. As melanopsin is not transported down the axon, IHC fails to identify it in central projections of these cells. In *Opn4-tau-LacZ* mutant mice (Pickard et al, 2009), the M1 cells and their projections are labeled with lacZ. These animals were injected with CTB, sacrificed one to two days later and the LGNs were sectioned and studied. ipRGC projections in the control animal (Figure 18A') show a crisp border at the edge of the dLGN, limiting them to the IGL and vLGN. IGL projections are diminished in the mutant and the border is less defined, with ipRGCs spilling into the dLGN and projecting out toward other thalamic nuclei (Figure 18B-B").

5) Image forming RGCs and their dLGN projections are normal in the reeler mutant

The above results demonstrate that non-image forming RGCs with abnormal projections to the LGN remain normal in the retina. We also addressed the targeting of image forming inputs. Calretinin expressing cells are known to project to the dLGN, bypassing the vLGN and IGL (Figure 19A). In order to ensure that targeting deficits in the absence of functional reelin were exclusive to the latter two subnuclei, these cells and their projections were studied in the retina and the LGN. Retinal cross sections were stained with a calretinin antibody. Stratification is clearly visible with no gross change in location of these cells and their arbors between the control (Figure 19B) and the mutant (figure 19C). LGN sections of these animals were stained with the same antibody. Here, in both the control animal (Figure 20A) and the mutant (Figure 20B), retinogeniculate projections are confined to the dLGN as expected. Although the border of this subnucleus

as it articulates with the IGL is altered in the mutant, there is no spill over of the calretinin stained axons out of it. This indicates that targeting defects seen in the *reeler* mutants do not affect RGCs projecting to the dLGN, as they do vLGN and IGL projections.

6) Layer VI corticothalamics to dLGN are normal in reeler

In addition to being targeted by different classes of retinal axons, LGN subnuclei receive different cortical inputs. Cortical layer VI neurons project to the dLGN (Figure 21A), and layer V to the vLGN and IGL. At present, there are no good tools to examine layer V projections in *reeler* mutants. To assess whether layer VI neurons correctly target the dLGN in the absence of functional reelin, we used the Golli-tau-GFP transgenic mouse, a mutant that fluorescently labels layer VI neurons and their corticothalamic axons (Jacobs et al, 2007). These mice were crossed with *reeler* heterozygotes to generate a transgenic:mutant colony. Breeding within this colony generated $reln^{+/+}$; golli-tau-GFP $^{+/?}$ controls and $reln^{rl/rl}$; golli-tau-GFP $^{+/?}$ mutants. It has been well established that cortical layers are disorganized in the *reeler* mutants (Katsuyama and Terashima, 2009). $Reln^{rl/rl}$; golli-tau-GFP $^{+/?}$ exhibited such layering defects (Figure 21C). Interestingly, the corticothalamic targeting to the dLGN was similar between controls (Figure 21D) and mutants (Figure 21E): projections were confined to the dLGN. These results show that despite cortical disorganization, corticothalamic targeting appears independent of neuron position in the cortex or reelin expression in the LGN.

7) *Cytoarchitecture of the LGN is normal in reeler*

Although previous results indicate that RGC targeting defects in the *reeler* LGN are the result of ineffective guidance, it is possible that the cytoarchitecture is improperly formed and responsible for the observed flaws. Immunohistochemical techniques were used to study other things known to be present in the LGN subnuclei. If the presence and location of these cells are unchanged in the absence of a functional reelin protein, it will lend support to the claim that reelin acts as a guidance cue for RGC axons targeting the vLGN and IGL.

The NeuN antibody is used as a neuronal marker. When applied to the control animals at various ages, a characteristic staining of cells throughout vLGN, IGL and dLGN was seen (Figure 22A). The mutant animal shows the same pattern (Figure 22B), with staining visible and consistent throughout the same two subnuclei. The cell density appears similar in the control and mutant LGNs. This demonstrates that neurons can, and do, develop in the LGN in the absence of a functional reelin protein.

Neuropeptide Y (NPY) is expressed by projection neurons in the IGL. Antibodies directed against this were used to establish the presence of these relay cells in the mutant. Both P7 control (Figure 22C) and *reeler* (Figure 22D) IGLs show strong immunoreactivity in the presence of the NPY antibody. The mutant IGL experienced the largest decrease in RGC projections in previous experiments, but does not show an absence of these resident NPY- expressing IGL relay neurons.

Glutamic acid decarboxylase (GAD 65) is an enzyme highly enriched in a large population of neurons in both the IGL and vLGN. Antibodies that are immunoreactive with processes of these cells were applied to brain sections containing the LGN. The pattern of fluorescence seen in the control LGN (Figure 22E) and the mutant (Figure 22F) are the same. GAD 65 is localized properly in both, with none seen incorrectly in the dLGN or other thalamic nuclei.

Vesicular glutamate transporter 2 (VGlut2) is known to be present at the terminals of retinogeniculate synapses in the dLGN(Land et al, 2004). Immunostaining in P17 control LGN confirmed its enrichment there. VGlut2 IHC in the mutant LGN appeared similar to controls indicating that VGLUT2 containing synapses of RGCs are not affected by the absence of a functional reelin.

Although there are other classes of cells that were not studied in the LGN, groups with large representation in each of the three subnuclei were found to be unchanged in the reeler mutant. This implies that the cytoarchitecture of this nucleus does not require a functional reelin protein in order to form properly. The failure of RGCs projecting to the vLGN and IGL to properly synapse in this mutant is likely due to a targeting defect and not a defect in the formation of the three LGN subnuclei.

Figure 9 Reelin protein expression is highest in vLGN and IGL at young ages

At P0 (**A**) reelin protein expression is the highest of all ages, and present almost exclusively in the vLGN and IGL. At P3 and P7 (**B, C**), when axons are still actively seeking targets, reelin is still enriched in the vLGN and IGL(**arrows**), but at decreasing levels when compared to P0. By P14 (**D**) very little reelin is seen in any part of the LGN.

vL= ventral lateral geniculate nucleus, dL= dorsal lateral geniculate nucleus, dashed lines define rough boundaries of each of the subnuclei

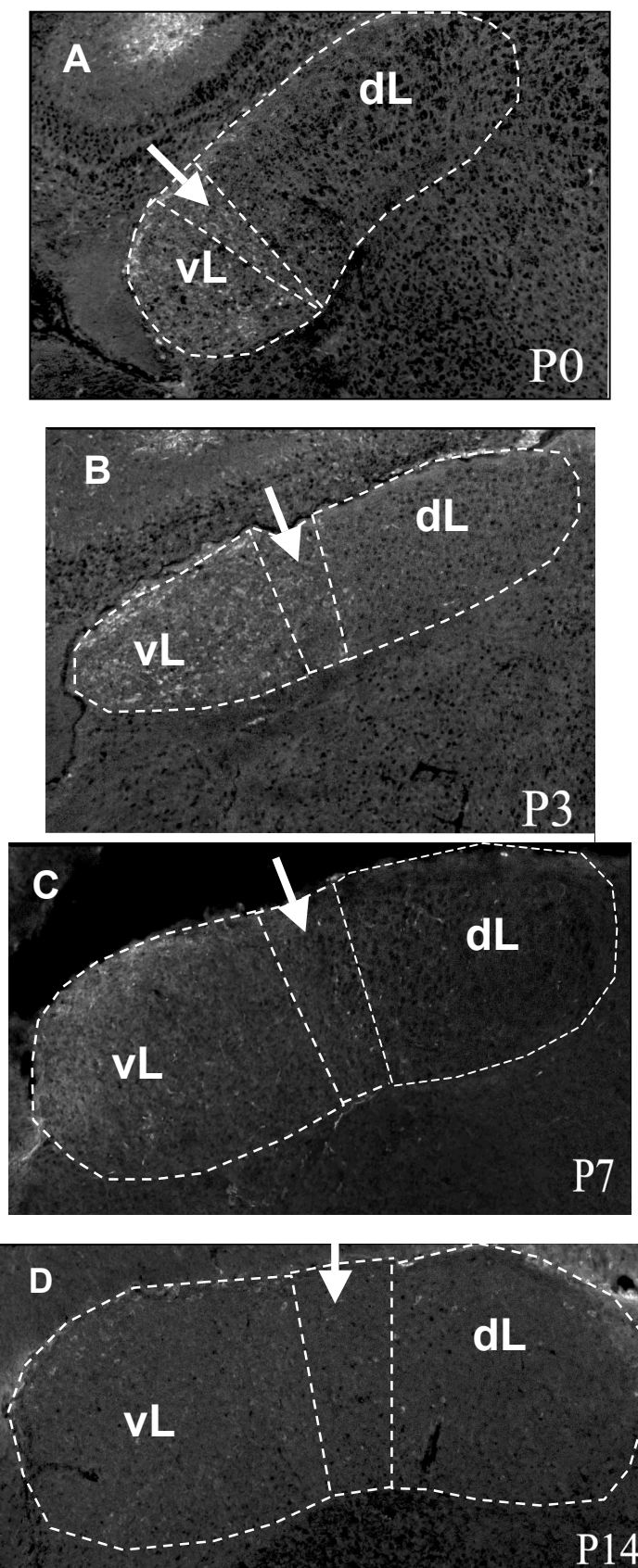


Figure 9 Reelin protein expression is highest in vLGN and IGL at young ages

Figure 10 P14 RGC projections to LGN

Gross defects are apparent in the targeting of retinal axons to the LGN in the absence of reelin (**B**) when compared with P14 controls (**A**). The projections to the IGL (**arrow**) appear much smaller in reeler mutants. The RGC synapses in mutant vLGN also make up a smaller area when compared with controls.

v= ventral lateral geniculate nucleus, d= dorsal lateral geniculate nucleus, arrows point to IGL

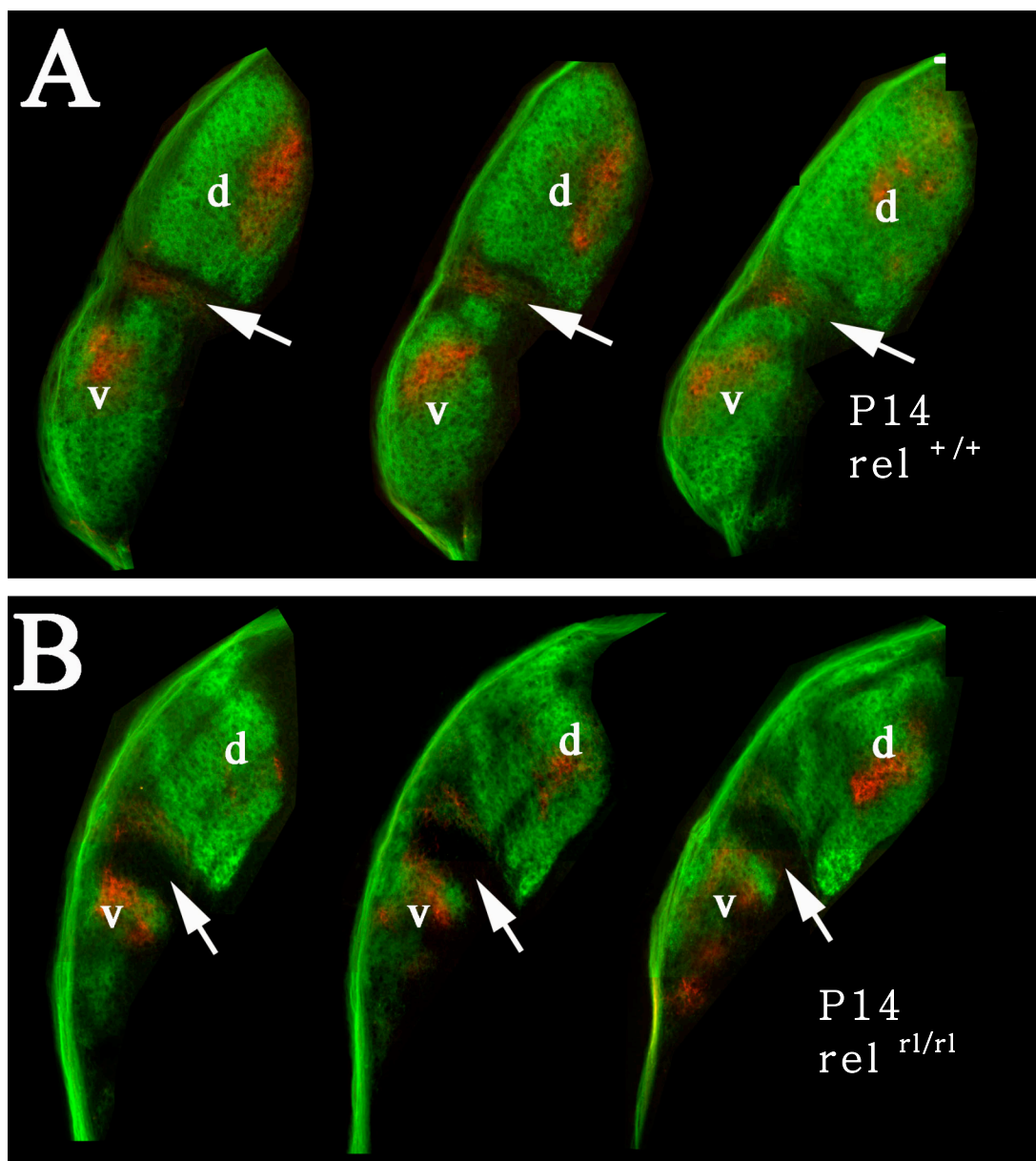


Figure 10 P14 RGC projections to LGN

Figure 11 Misrouting of axons away from reeler mutant LGN

Labeled axons from the IGL of mutant mouse are misrouted, projecting toward incorrect thalamic nuclei.

dL= dorsal lateral geniculate nucleus, vL= ventral lateral geniculate nucleus

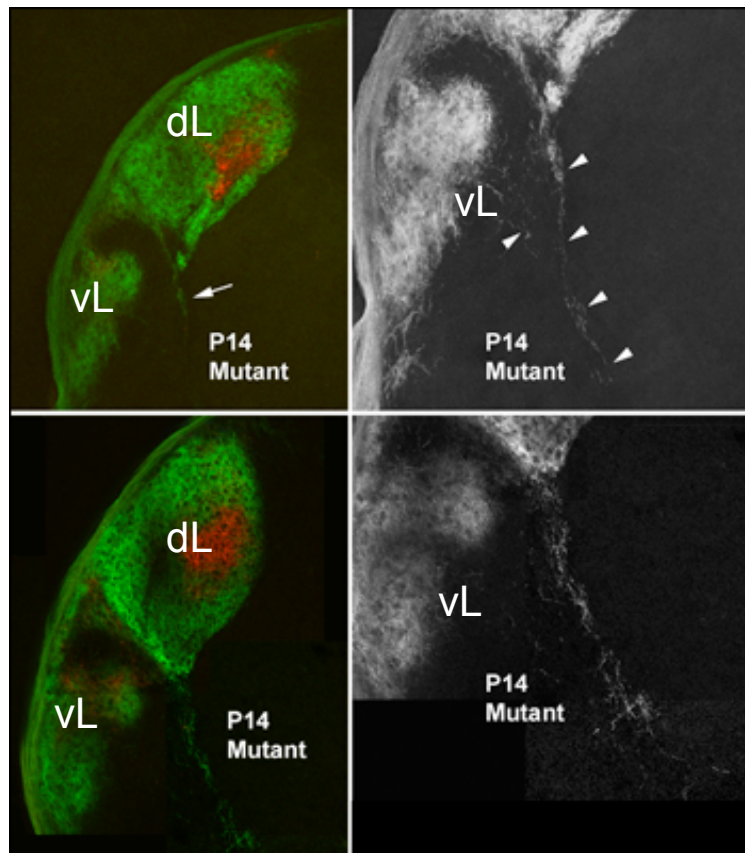


Figure 11 Misrouting of axons away from reeler mutant LGN

Figure 12 Rostral to Caudal Series of Wild Type and Mutant LGN

P14 control (A, A') and reeler LGNs (B, B') were sectioned from rostral to caudal ends and aligned in a series. The decreases in vLGN and IGL projections are seen in all sections.

P7 wild type (C) and reeler LGNs (C') show the same changes in RGC projections.

d= dorsal lateral geniculate nucleus, i= intergeniculate leaflet, v= ventral lateral geniculate nucleus

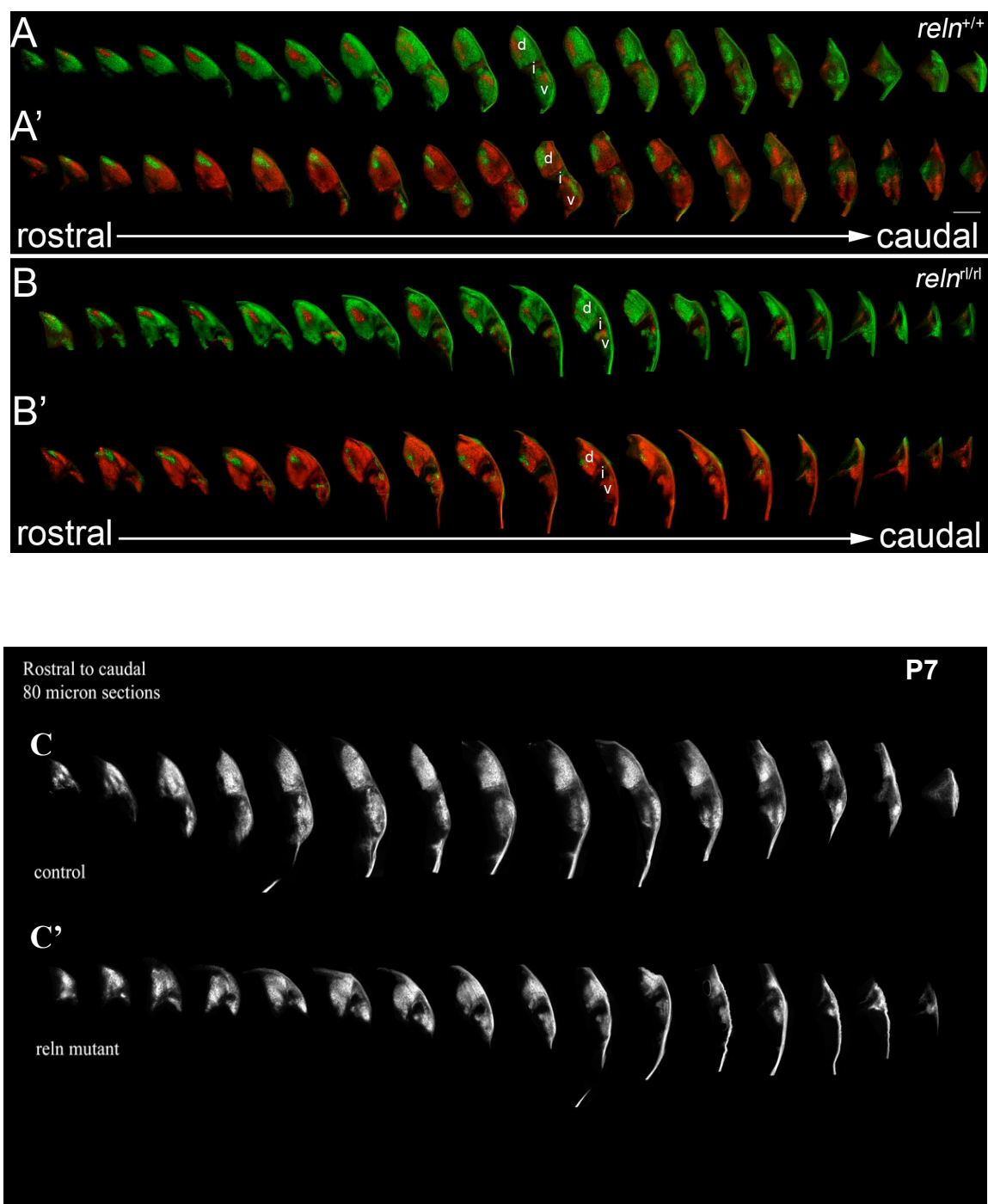


Figure 12 Rostral to Caudal Series of Wild Type and Mutant LGN

Figure 13 Mutant RGC projections to LGN compared to control

Using the control LGN as the standard, the relative percentage of the area occupied by RGC projections in the vLGN, IGL and dLGN in the mutants and controls were studied. The mutant IGL showed the greatest decrease in the relative area of retinal projections, followed by the vLGN. The percentage of projections to the dLGN, relative to the control, appeared to increase. (n=6)

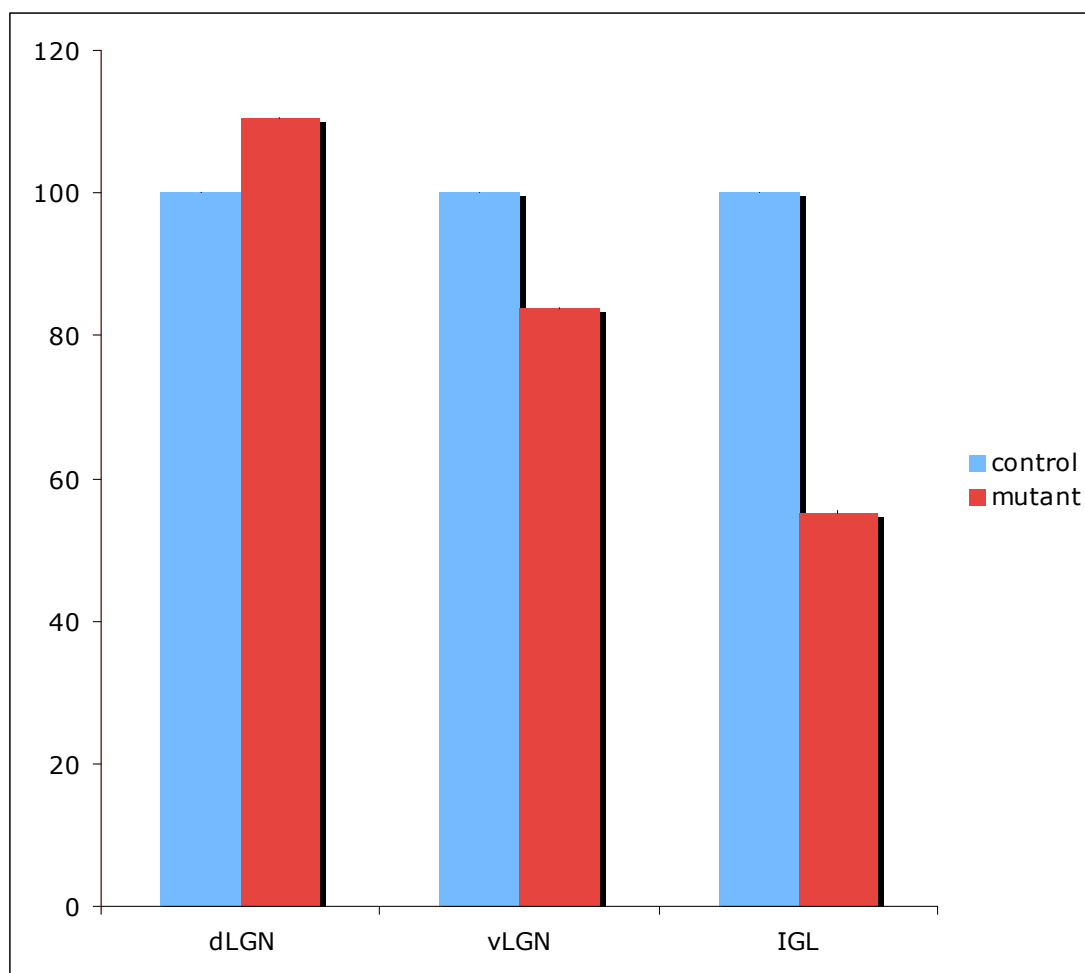


Figure 13 Mutant RGC projections to LGN compared to control

Figure 14 P1 retinogeniculate projections are altered in the reeler mutant

As early as P1, projections to the vLGN and IGL are visibly smaller in the mutant LGN **(B)**, and the dLGN projections appear larger than in the control **(A)**, relative to the other two subnuclei.

Aberrant projections, not seen in the P1 control **(C)**, project from the IGL/dLGN border into the thalamus in the mutant **(D, D')**.

d= dorsal lateral geniculate nucleus, v= ventral lateral geniculate nucleus, arrows=
intergeniculate leaflet

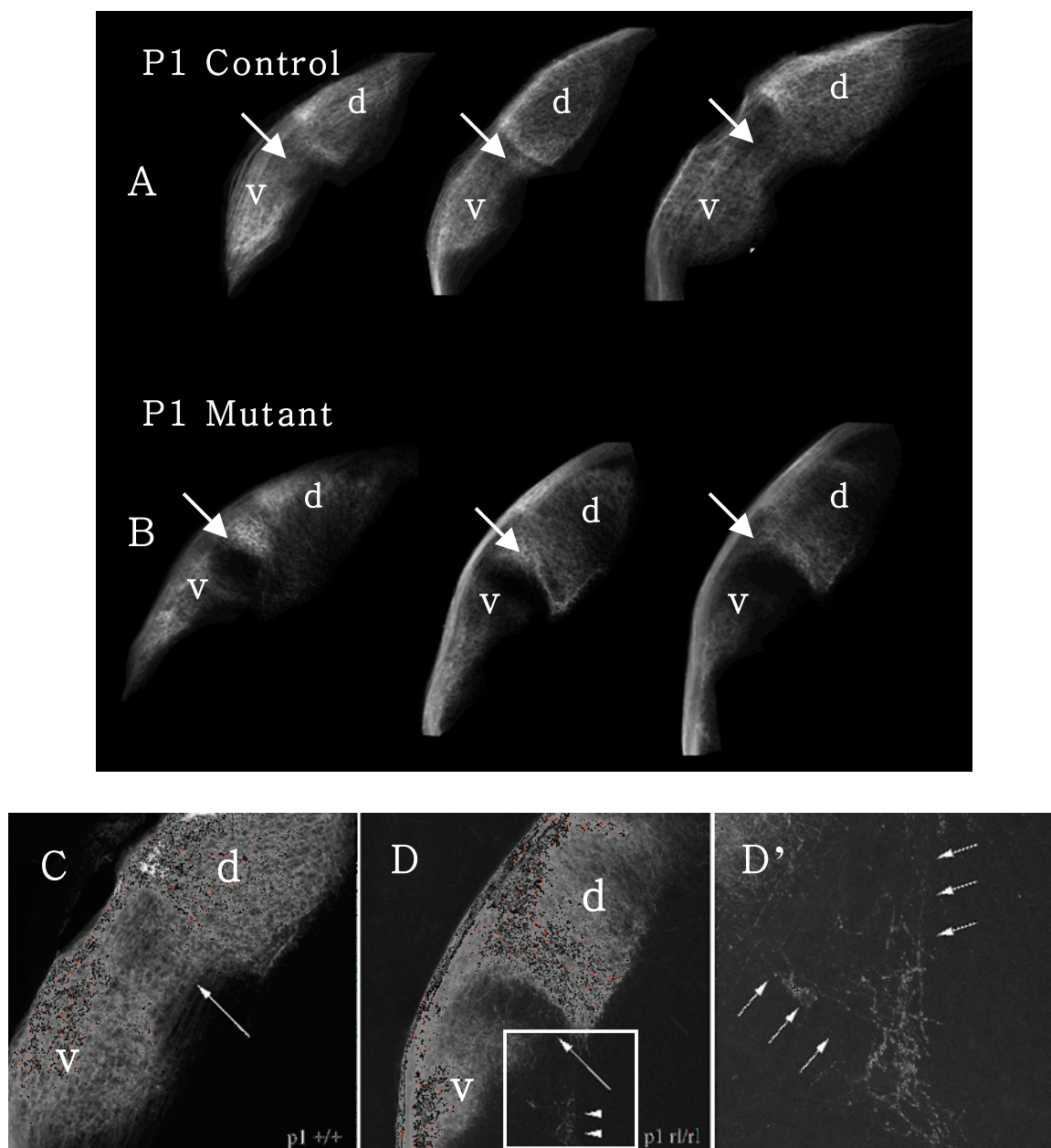


Figure 14 P1 retinogeniculate projections are altered in the reeler mutant

Figure 15 Melanopsin expressing intrinsically photosensitive retinal ganglion cells (ipRGCs), appear normal in the absence of reelin

Within the LGN, ipRGCs project only to the vLGN and IGL (**A**) and do not target the dLGN. Whole mounts of P12 retinas (**B**) do not display any gross morphological differences. Mutant cells (also stained with melanopsin) are able to take up CTB (**B'**), indicating that there is no damage to the soma.

Axons of both P10 control (**C**) and mutants (**D**) extend toward the optic disc (**arrows**)

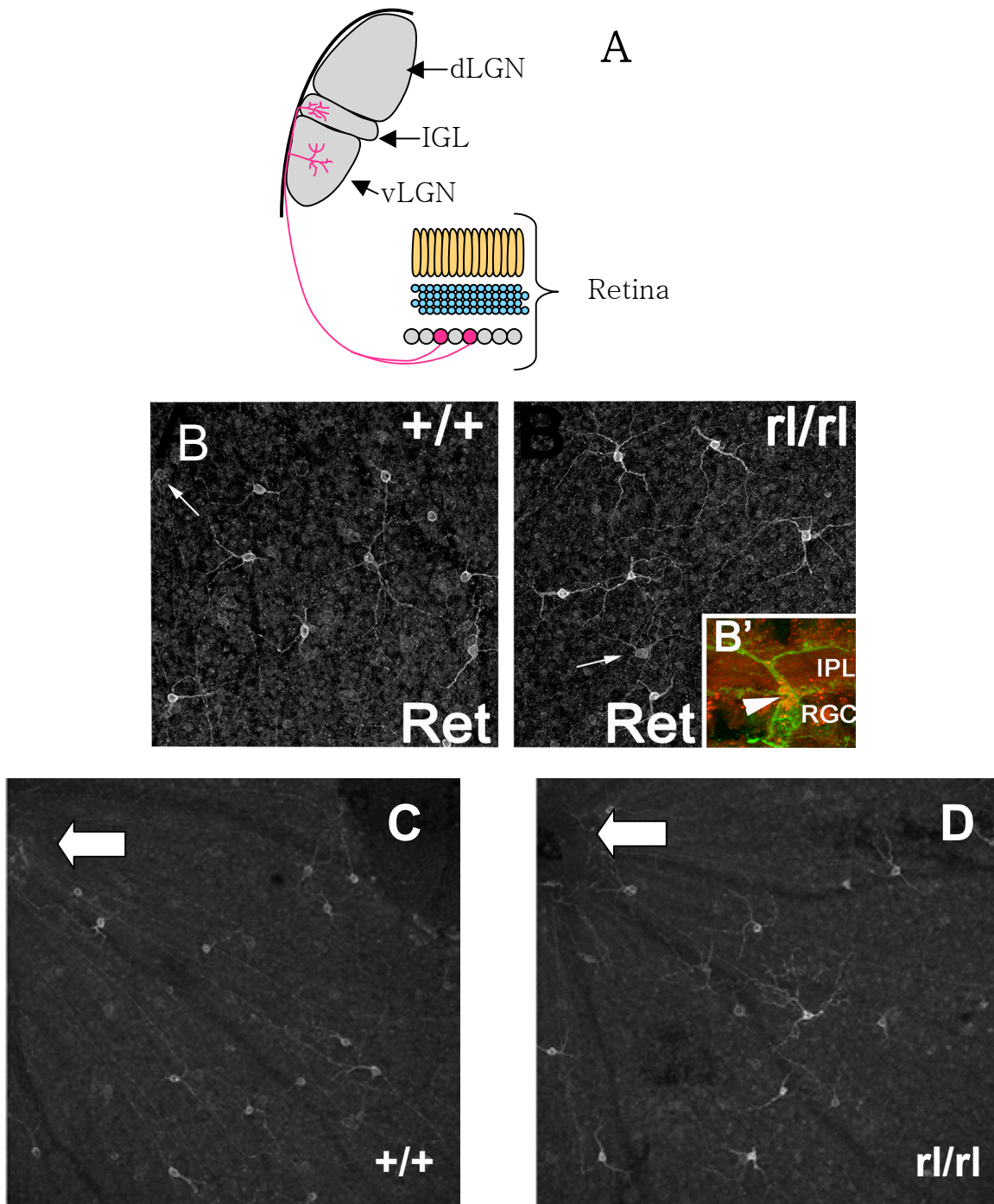


Figure 15 Melanopsin expressing intrinsically photosensitive retinal ganglion cells (ipRGCs), appear normal in the absence of reelin

Figure 16 Melanopsin expressing ipRGCs display normal morphology in the absence of reelin

No differences were noted between melanopsin stained retinas of the control **(A)** and the mutant **(B)**. In both, cell bodies of ipRGCs remain in the ganglion cell layer **(GCL)** with dendritic arborization along either border of the inner plexiform layer **(IPL)**.

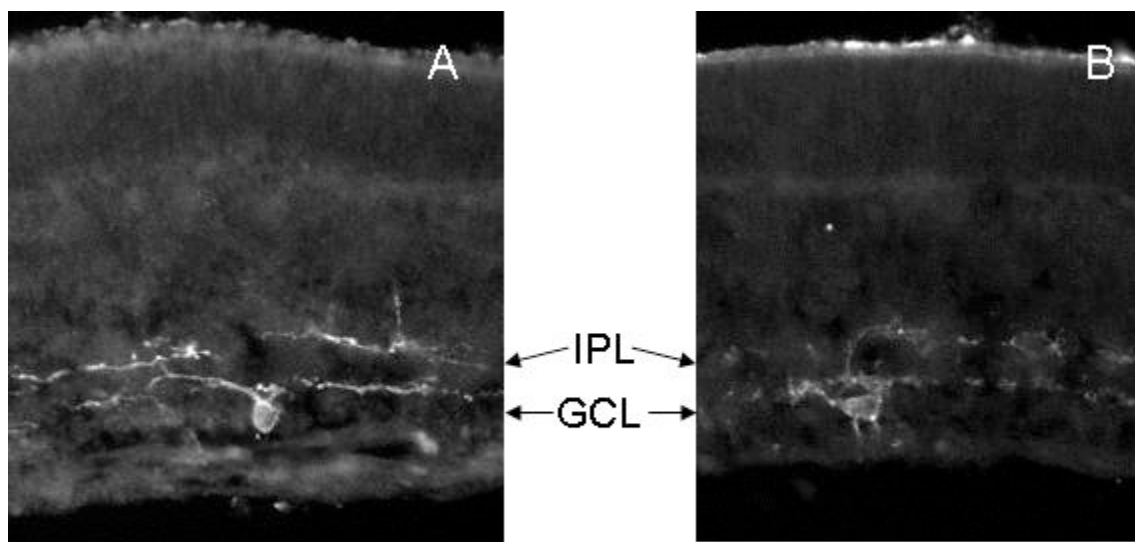


Figure 16 Melanopsin expressing ipRGCS display normal morphology in the absence of reelin

Figure 17 RGC projections to the Olivary Pretectal Nucleus (OPN) and Suprachiasmatic Nucleus(SCN) appear normal in the reeler mutant

Opn4-tau-LacZ mice, that label M1 RGCs and their projections with LacZ, were crossed with reeler mutants and received intraocular injections of CTB. The observed projections to the OPN in the $reln^{+/+}$ control(**A**) show no gross differences when compared to those in the $reln^{rl/rl}$ mutant (**A'**).

Comparing the same animals, no defects were observed in the targeting of retinal axons to the control SCN (**B**) and the mutant (**B'**).

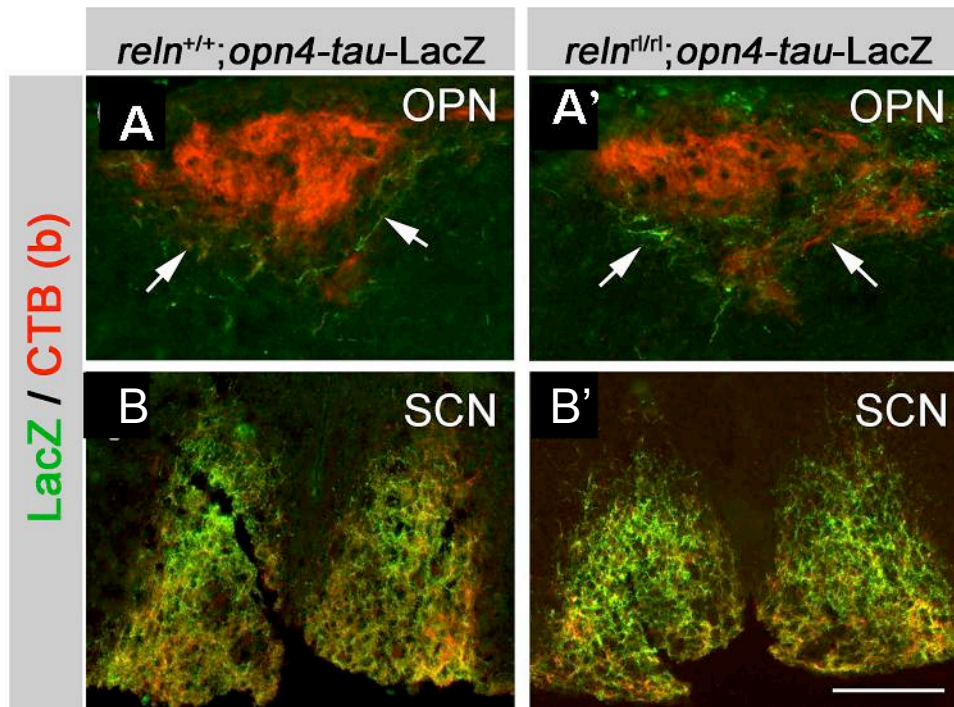


Figure 17 RGC projections to the Olivary Pretectal Nucleus (OPN) and Suprachiasmatic Nucleus(SCN) appear normal in the reeler mutant

Figure 18: M1 retinogeniculate projections are altered in the absence of reelin

These animals are mutated so that M1 ipRGCs and their projections express LacZ, which is detected by IHC (**green**). They were co-stained using CTB injections (**red**) to highlight all retinal projections. In the wild-type animal (**A**), M1 projections are clearly limited to the vLGN and IGL, with a crisp border at the dLGN. In the reeler mutant (**B**), these projections spill incorrectly into the dLGN with aberrant axons heading toward other thalamic nuclei (**C**).

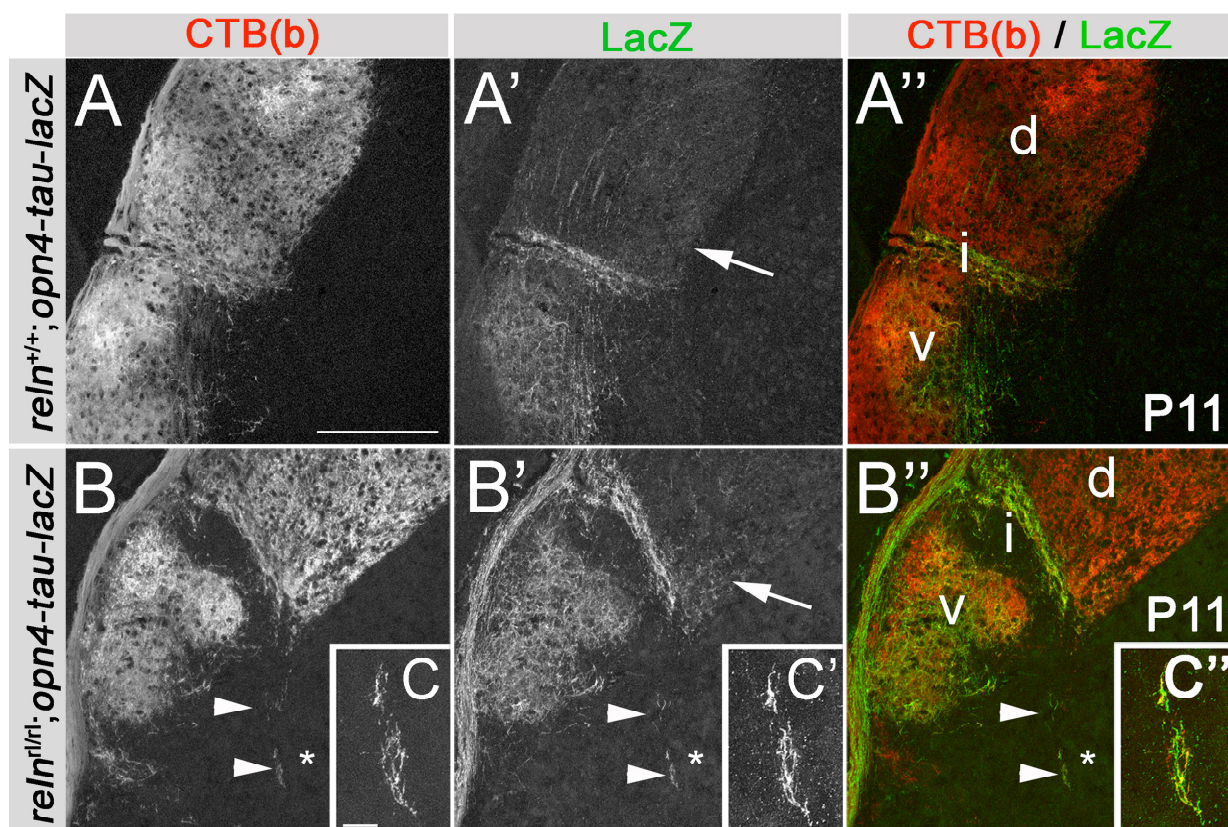


Figure 18: M1 retinogeniculate projections are altered in the absence of reelin

Figure 19: Calretinin expressing, image-forming RGCs, appear normal in the absence of reelin

There are 10 classes of RGCs that project only to the dLGN, bypassing the vLGN and IGL (**A**). Staining with antibodies directed against calretinin, no gross difference are seen between the wild-type retina (**B**) and the mutant (**C**).

dLGN= dorsal lateral geniculate nucleus, IGL= intergeniculate leaflet, vLGN= ventral lateral geniculate nucleus, INL= inner nuclear layer, IPL= inner plexiform layer, GCL= ganglion cell layer

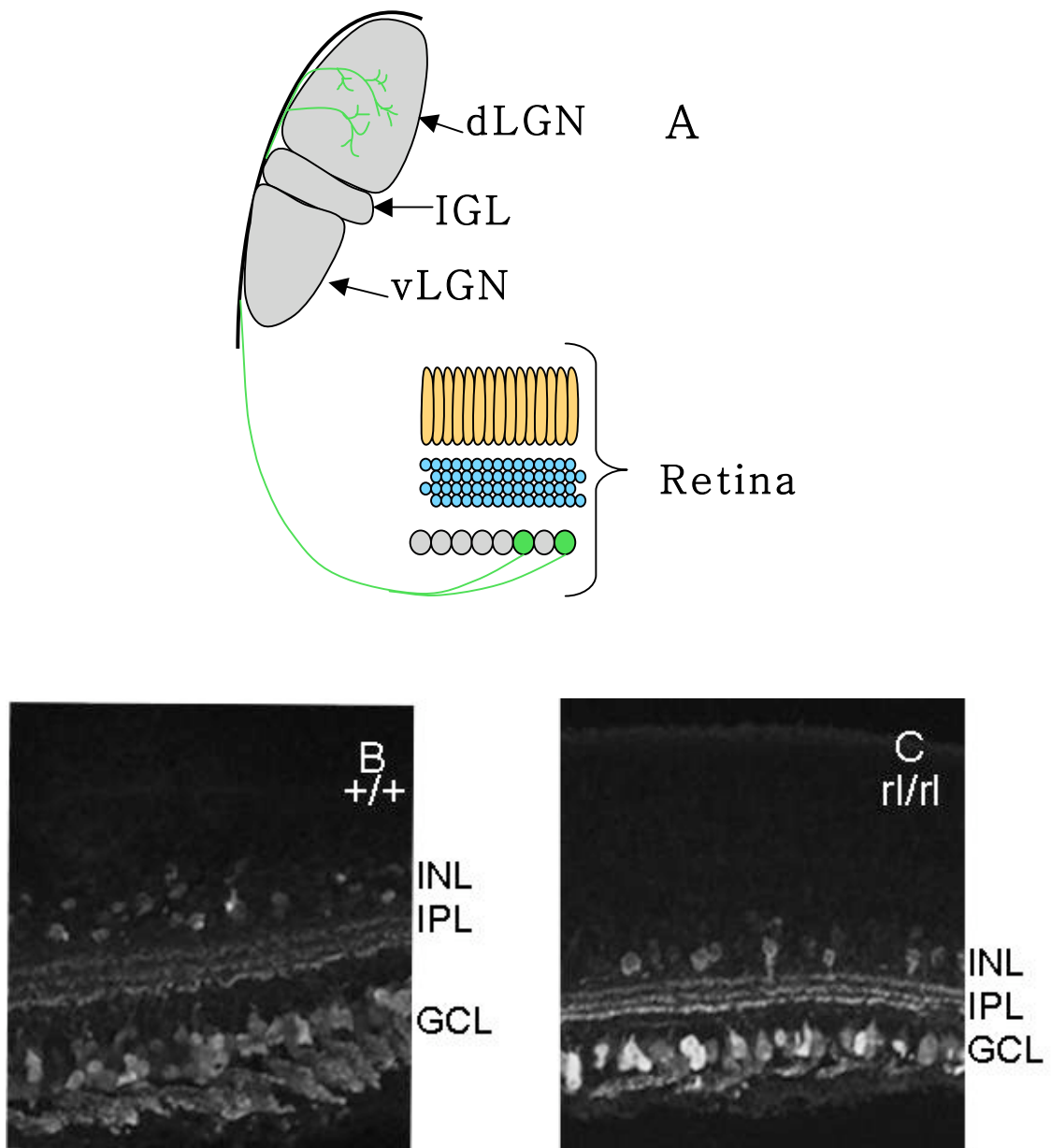


Figure 19: Calretinin expressing, image-forming RGCs, appear normal in the absence of reelin

Figure 20 Calretinin expressing RGCs normally target the dLGN in reeler mutants

Antibodies directed against Calretinin were used to highlight the projections of RGCs to the LGN. In the wild-type (**A**) and the mutant animal (**B**), all projections were limited to the dLGN (**arrows**). There was no incorrect targeting the the vLGN, IGL or other thalamic nuclei in the reeler mutant.

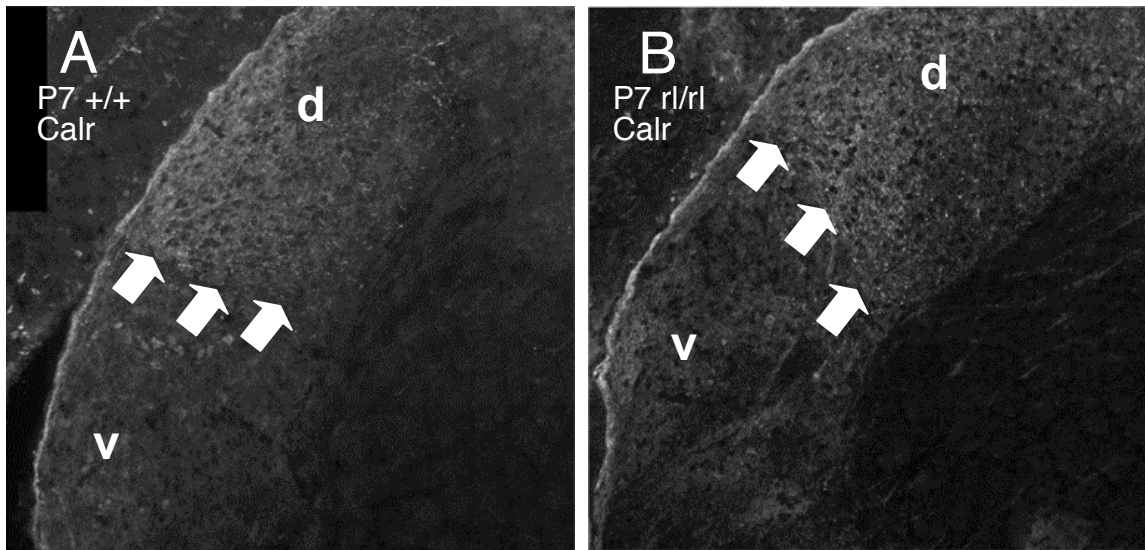


Figure 20 Calretinin expressing RGCs normally target the dLGN in reeler mutants

Figure 21 Corticothalamics originating from Layer VI correctly target dLGN in reeler mutants

The Golli-tau-GFP transgenic mouse labels layer VI cortical neurons and their axons.

These projection to the dLGN, bypassing the vLGN and IGL (**A**). These animals were crossed with $reln^{+/+}$ controls and $reln^{rl/rl}$ mutants.

The cortex in the control (**B**) showed proper confinement of layer VI neurons, the mutant showed migration of these to incorrect cortical layers (**C**). The projections of both, however, were correctly guided to the dLGN with no faulty corticothalamics targeting the vLGN or IGL (**D,E**).

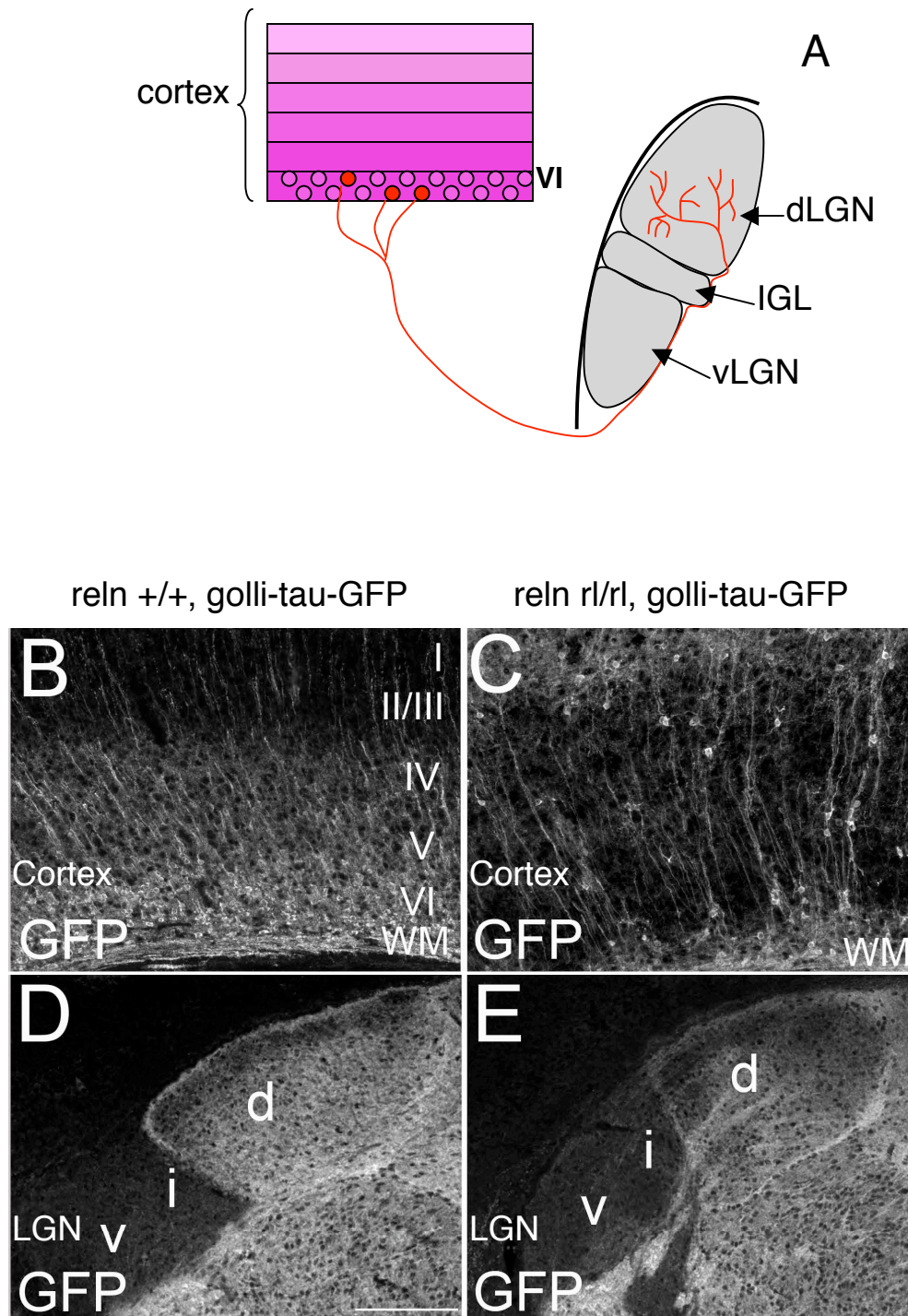


Figure 21 Corticothalamics originating from Layer VI correctly target dLGN in *reeler* mutants

Figure 22 Immunohistochemistry of P17 LGN Cytoarchitecture

Anti-NeuN antibodies were used to locate neuronal cell bodies. In both the control **(A)** and the mutant LGN **(B)**, neurons were found in all three subnuclei with no gross differences in morphology or distribution.

Anti-NPY antibodies are immunoreactive with relay neurons in the IGL. These were present in both the control **(C)** and the mutant **(D)**.

GAD65, only present in vLGN and IGL, was correctly localized in both the control **(E)** and the mutant **(F)** with none seen in the dLGN.

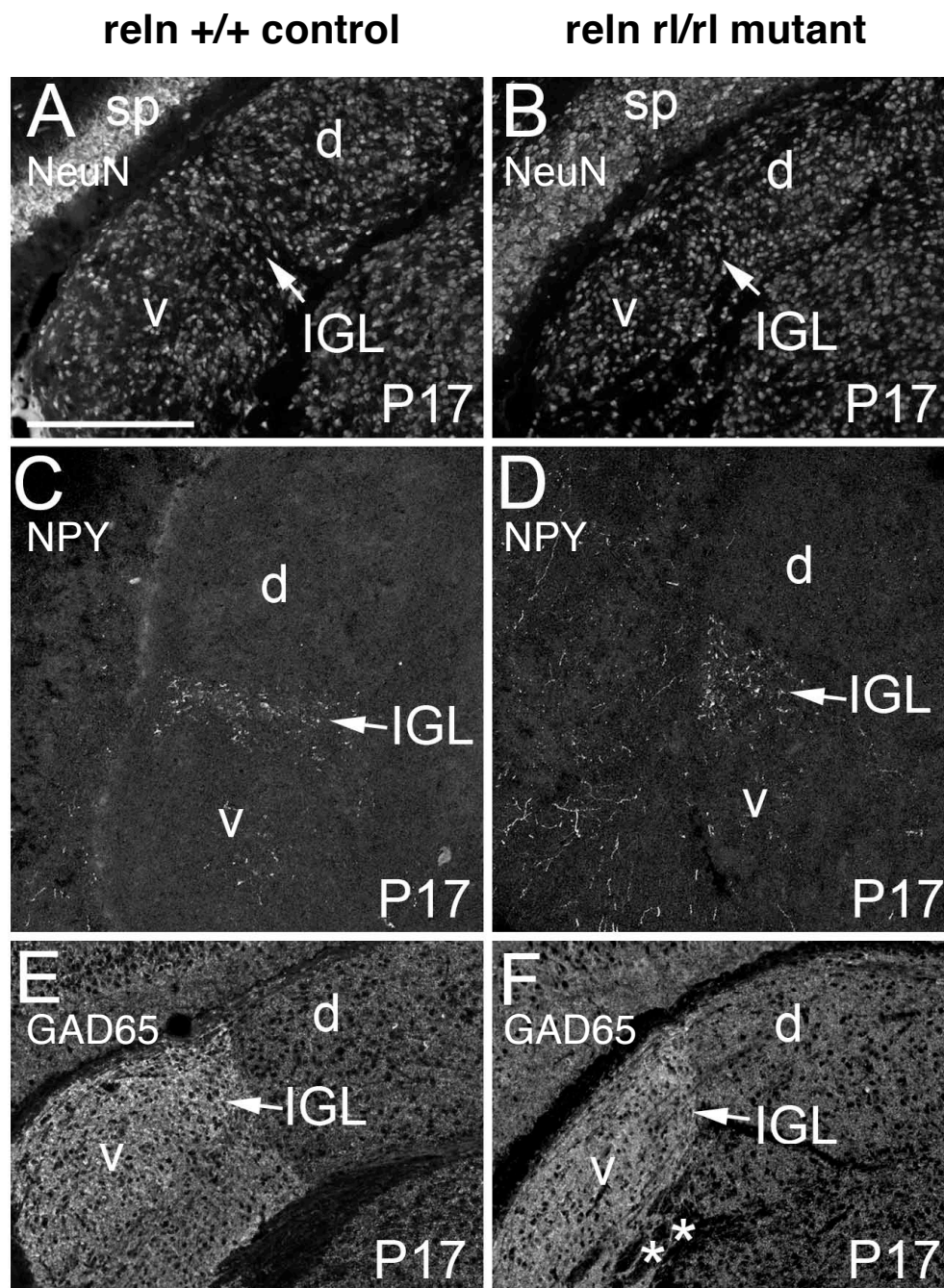


Figure 22 Immunohistochemistry of P17 LGN Cytoarchitecture

DISCUSSION

Cells in the CNS often convey information to post-synaptic partners found a great distance away. In order to do this, the pre-synaptic neurons must extend axons over these distances to very specific target neurons. This process is extremely complicated and precise. Attractive and repulsive cues, both extracellular and cell membrane bound, meet the axon at various steps of the journey to direct it on its course. Upsetting the expression of these molecules leads to the formation of incorrect neuronal circuitry and nervous system dysfunction, making their study important to researchers.

The central projections of the visual system make an excellent model for examining this type of targeting in the brain. The eye is easily manipulated by the researcher, making the visual system well studied. The retinal ganglion cells (RGCs), which are the eye's communication with the brain, show great specificity in their synaptic patterns.

Individual morphologically classified groups of these cells display a specificity of input that is conveyed in the nuclei they target. Image forming input will be sent by some classes of RGCs to the dorsal lateral geniculate nucleus (dLGN) exclusively, destined for processing in the primary visual cortex. Non-image forming stimuli, responsible for functions such as pupillary reactions and circadian rhythm, activate exclusive cells in the retina and are carried through wholly different pathways. These will project to the ventral LGN, intergeniculate leaflet, suprachiasmatic nucleus and olivary pretectal nucleus; but none will communicate with the dLGN or the visual cortex. The non-image forming nuclei share reciprocal connections amongst themselves to control a host of functions.

The manner in which RGC axons find the correct post-synaptic partner to communicate with is an example of class-specific targeting.

The mechanisms controlling the formation of many visual circuits have been extensively documented. Specificity in the segregation of RGC axons to subnuclei once they reach the LGN has not been well studied. The dLGN processes information that is functionally distinct from the vLGN and IGL, so the axons that navigate to each of these are different and require divergent guidance cues. In order to identify molecules that may be responsible for targeting LGN subnuclei, microarray analysis was performed on tissue pooled from either the vLGN and IGL or the dLGN in P3 wild-type animals. mRNA that showed enrichment in the vLGN and IGL was examined further using qPCR to identify patterns of expression at progressive stages of development. The expression of one gene, *reln*, was found to match what was expected of a targeting cue. It was significantly higher in the vLGN and the IGL compared with the dLGN, and expression was high as retinal axons arrived then reduced when they no longer were seeking targets. Thus, *reln* became a likely candidate for class-specific retinogeniculate guidance to the vLGN and IGL.

Studies performed here looked at the location of reelin, the protein produced by the *reln* gene, using immunohistochemistry (IHC). It was found to be enriched in the vLGN and IGL in young brains, with expression decreasing as the animals aged. This suggests a role for reelin in the targeting of extending RGC axons in non-image forming circuitry development. Animals with a mutated *reln* gene were used to examine the effects of a non-functioning reelin protein on retinogeniculate development. Cholera toxin B (CTB),

which is taken up by RGC bodies and transported down their axons, was injected into the eye to serve as an anterograde tracer to locate projections to the LGN. Sectioned brains of these animals showed a decrease in projections to the vLGN and IGL, when compared to littermate controls, and aberrant RGC axons venturing into incorrect nuclei in the thalamus. No change was observed at any age in dLGN. Defects were seen as early as P1, confirming that the reelin protein is required for correct initial targeting of non-image forming retinal circuitry within the LGN.

Unsure whether the targeting defects seen in the *reeler* mutant were initiated at the retinal level or once axons reached the thalamus, retinal cells known to project to different LGN subnuclei were studied. Melanopsin expressing, intrinsically photosensitive RGCs (ipRGCs) carry non-image forming input to the vLGN, IGL and their reciprocal targets. Antibodies against these cells showed normal cell body morphology and arborization in the retina without a functional reelin present. The projections of these cells to the vLGN and IGL were altered in the manner already discussed, with an overall decrease and with tracts incorrectly venturing into the thalamus. Projections of the same class of cells to the OPN and SCN appeared unaffected by the mutation. These results further support our claim that reelin acts on class-specific guidance at the LGN.

Studies of known cortical *reeler* defects and their corticothalamic projections to the dLGN are worth noting, as well. It has been well established that this mutant presents with alterations in cortical lamination (Katsuyama and Terashima, 2009). We saw this in our own experiments looking at layer VI cortical neurons, where cell bodies were

improperly localized throughout the cortex. Their projections to the dLGN, however, were unaffected. A crisp border was noted between the dLGN and IGL, with no obvious faulty projections into any other thalamic nuclei. Again, the functional reelin protein is not required for targeting of axons seeking the dLGN. Most surprisingly, cortical cells displaying obvious defects in cortical lamination possess corticothalamics that are still capable of reaching proper targets in the brain.

Many non-LGN areas affected in the *reeler* mutant display alterations in cytoarchitecture. The cortex, cerebellum, hippocampus and superior colliculus, all laminated structures, experience changes in layering when the functional reelin protein is not available (Rice and Curran, 2001; Katsuyama and Terashima, 2009; Borrell et al, 2007). The cytoarchitecture of the *reeler* LGN was examined using IHC. NeuN staining demonstrated that neurons were appropriately positioned in all three subnuclei of the mutant, as opposed to the overlying hippocampal structures. Antibodies against NPY found relay neurons present in the IGL, VGlut2 established that synaptic terminals remained enriched in the dLGN and GAD65 expression in the vLGN and IGL were normal. Essentially, IHC studies done found the cytoarchitecture in the LGN to be unaffected by a faulty reelin protein. This is unusual for the *reeler*, as most areas affected by the mutation experience some degree of disruption of cell order. Considering the obvious defects seen in vLGN and IGL retinogeniculates, it appears reelin is only responsible for their targeting within this region. It is still possible that other cytoarchitectural defects that were not sought exist, and that they affect the targeting.

Knowing the role that reelin plays in retinogeniculate targeting, we are still unsure exactly how it performs the task. It has been well established that reelin is a large secreted glycoprotein that binds with membrane bound very low density lipoprotein receptor (VLDLR) and apolipoprotein ER2 (ApoER2) (Rice and Curran, 2001). Animals containing knockouts of either or both of these receptors present with phenotypes that, in part, mimic the *reeler* phenotype (Tromsdorff et al, 1999). Once bound, these receptors homodimerize and bind cytosolic Disabled1 (Dab1) to initiate intracellular pathways that effect migration via cytoskeletal arrangement. Mice deficient in Dab1 share anatomical, functional and behavioral defects observed in reeler mutants (Borrell et al, 2007; Sheldon et al, 2007). Dab1 has been shown to be necessary for reelin's role in neuronal migration, axon guidance and targeting, and synaptic function. Therefore it seems likely that Dab1 and reelin receptors, are required for reelin's role in retinogeniculate targeting.

At this point, we have yet to discover a cytoarchitectural deficit similar to lamination changes in other regions that could be linked to defects seen in the reeler LGN. It is still possible that one exists, and acts upon retinogeniculate axons as they approach. However, it is at least clear that such a defect does not alter the formation of the three distinct LGN subnuclei. More subtle defects in cytoarchitecture, perhaps in dendritic arborization in the vLGN and IGL, could certainly affect axonal targeting in reeler mutants. This can be easily envisioned in the mutant IGL, but is more difficult to imagine for retinal axons that are misrouted up to, or more than, one millimeter into inappropriate thalamic regions.

Immunostaining for reelin in the retina was not performed, but it has been found in multiple layers by other researchers. The IPL, ganglion cells in the GCL and amacrine cells all express reelin; which plays a role in synaptic circuit formation here. Dab1, a downstream component of the reelin pathway, has also been localized to amacrine cells, the IPL and the GCL border (Rice et al, 2001b). It is known to regulate neuronal positioning in the fly retina (Pramatorova et al, 2006). Although we were unable to visualize any deficits in the reeler retina based on the cells that we studied, other studies have found that targeting and functional defects exist (Rice et al, 2001b). While it is possible that targeting defects in retinogeniculate projections are secondary to cytoarchitectural changes in the retina; we feel it is unlikely as other CNS targets of these RGC axons, the OPN and SCN, appear normal in mutants.

The altered lamination in *reeler* brain regions is due to faulty migration of cells in the developing nervous system. We propose that, in the absence of these cytoarchitectural changes in the LGN, reelin may serve as an extracellular axon guidance cue in the LGN. Aside from well documented roles in direction of cell migration, reelin has been found to be a guidance cue in the hippocampus and cortex (Del Rio et al, 1997; Frotscher et al, 1997). These areas are known to exhibit both migration and axon navigation defects in the absence of a functional reelin protein. Our results suggest that the latter is true in retinogeniculate projections seeking the vLGN and IGL as reelin was found to be present in these areas during targeting, and axons seeking them were misrouted upon arrival.

Reelin may not act independently in this guidance. Alternatively, reelin is a large glycoprotein and may assist in creating an extracellular environment that suits other attractive or repulsive cues. Its functional absence may upset the actions of these. Many other molecules were found in these same regions by microarray analysis. While further studies to determine the location of their protein expression in the LGN was not done, some of these may also be involved in this guidance. Sema3c is part of the semaphorin family, known to participate in many aspects of CNS development and to have its effects mediated by other cues (Erskine and Herrera, 2007). Sema3D is present at the chiasm and promotes crossing of contralateral projections (Sakai and Halloran, 2006) and takes part in mapping of the tectum (Liu et al, 2004). The eye specific crossing at the chiasm and retinotopic mapping in the tectum could suggest that Semas may act in other forms of targeting specificity, such as the class specific seen in the LGN. The action of some semaphorins occurs in concert with receptor complexes formed by neuropilins and CAMs (Castellani et al, 2002; Falk et al, 2005). Interactions between semaphorins and reelin have not been investigated. However, the presence of the two in levels that vary with development may be due to a combined control of retinogeniculate targeting.

Slit2 was also found to be present in significantly higher amounts in the vLGN and IGL than in the dLGN in the developing brain. Slits, like semaphorins, are well studied as guidance cues active in various parts of the brain. Slit1 and Slit2, present in the retina, guide RGCs via their inhibitory interactions with Robos on their growth cones (Erskine et al, 2000; Niclou et al, 2000). They also play inhibitory roles at the chiasm (Plump et al., 2002) and in the optic tract (Thompson et al, 2006). Via microarray and qPCR, it was

discovered that slit expression in the non-image forming LGN subnuclei was steady through development. The traditionally inhibitory action of this molecule may be important for keeping axons destined for other regions from landing in the vLGN and IGL. In the absence of the functional reelin, it was found that some axons meant for these subnuclei exited incorrectly into the thalamus. Perhaps slit2 plays a role in restriction here that is disrupted without a functioning reelin.

Based on our findings, it appears that reelin plays an attractive role to RGC axons seeking the vLGN and IGL. However, the interactions of signaling cues and their growth cone receptors are not simple. In some cases, varying levels of a molecule can change the way it directs its ligand. Some signaling molecules, such as netrin, have been found to perform both attraction and repulsion duties (Chisholm and Tessier-Lavigne, 1999). Changes in the approaching axons receptors can lead to multiple pathway choices. Slit-Robo interactions have been found to be attractive in some instances, rather than repulsive, based on the Robo receptors location in the growth cone (Englund et al, 2002). This same phenomenon has been observed in Wnts and netrins in the visual system, where axons can essentially select a direction for extension (Killeen and Sybingco, 2008). Claiming that the reelin-RGC axon exchange is one of simple attraction would be misguided, as more studies would need to be done to rule out multiple functions for the protein in guidance.

Further phenotypical ramifications of the ill formed LGN are worth studying. We know that reeler mice develop poorly, die younger than littermates and present with the

characteristic reeling gait and tremor. However, the vLGN and IGL receive input that participates in many non-image forming circuits. These subnuclei of the LGN, in cohort with other non-thalamic nuclei, contribute to the pupillary light reflex and circadian rhythms. While lesions in these areas have been studied (Harrington, 1997), the effects of a non-functional reelin on the development of these pathways is unknown and warrant attention.

While our findings are interesting, many more aspects of reelin's role in retinogeniculate targeting are worth exploring. We can say with confidence that the absence of a functional reelin protein has an effect on class-specific targeting to the vLGN and IGL, but how it does so is still not understood. Based on our results, a role in retinogeniculate guidance is implicated, but not in cell migration. Defects affecting the retinal circuitry and cell morphology may alter the course of RGC axons before they leave the eye. However, OPN and SCN projections of ipRGC's don't appear affected, nor do image forming retinogeniculates. The reasons why the defective reelin only affects vLGN and IGL guidance must be explored. There are many other molecules present in the vLGN and IGL that may act as cohorts with reelin, both in attraction to or restriction within these regions. Repulsive cues from the dLGN must remain intact in the reeler mutant, as few axons incorrectly target this subnucleus. These have not been identified, but may help to illustrate the entire profile of cues required for class-specific LGN targeting. Still more cytoarchitectural studies should be done, as alterations here may play a role in the misrouting of axons. All of these aspects of guidance, and potential changes in them that

arise in the *reeler* mutant, should be studied to gain further insight into reelin's newly established role in class-specific retinogeniculate targeting.

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